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IMMUNOGENETIC STUDIES OF SPECIES: SEGREGATION OF SERUM COMPONENTS IN BACK-CROSS INDIVIDUALS[1]

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ONE of the most specific means of analyzing relationships among species is the assay of the similarity of their proteins by precipitin reactions. Such a method was first extensively used by NUTTALL (1904), and following his work, many investigators have used immunological technics in comparable studies. Usually the criterion for distinguishing species by the precipitin test has been of a quantitative nature—that is, in terms of differences in the ability of the antiserum to interact with varying dilutions of the antigen, or by measuring the amounts of the precipitate produced. Qualitative differences in the proteins of closely related species (that is, the reaction of a reagent with the serum of one species and not with that of the other) have seldom been reported, probably because of difficulties inherent in the technic of precipitin-absorption, as pointed out by LANDSTEINER and VAN DER SCHEER (1924). Reference to papers on this general subject have been cited by LANDSTEINER and VAN DER SCHEER (1940) and CUMLEY and IRWIN (1940).

Relatively few studies have been made of the qualitative interrelationships of the sera of species and species hybrids. SASAKI (1937) reported that, following the absorption of antisera (precipitins) against the sera of Leghorns (*Gallus domesticus*) and guinea fowl (*Numida meleagris*), respectively, by the serum of the other, the serum of each species was sharply differentiated from that of the other—that is, each species possessed species-specific components in its serum. Furthermore, the serum of the hybrid between these two species possessed some, perhaps all, of the antigenic constituents of both parents. According to SASAKI (1935), similar results had been obtained previously by ISHIHARA and MISAO (1928) in their studies of carp and by SASAKI (1926) in an investigation of a species cross in ducks.

In a previous report (CUMLEY and IRWIN 1940) it was shown that qualitative differences exist in the sera of Pearlneck (*Streptopelia chinensis*) and Ring dove (*St. risoria*). The serum of the species hybrid possessed all or nearly all the species-specific and common properties of both parents.

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Related studies have been made by BROWN (cited by LOEB 1917) on hemoglobin crystals of the bloods of the horse, donkey, and mule and by MORITZ (1934) for the proteins of some plant hybrids.

The findings that the various species hybrids contain in their sera at least a part of the antigenic components of the sera of both parental species, parallel those on the relationship of the cellular characters of different species hybrids and their respective parental species, as reported from this laboratory (IRWIN and COLE 1936a, 1936b; IRWIN et al. 1936). Furthermore, the cellular characters which differentiate one species from another have been found to be gene-determined, as shown by their segregation in backcross progeny (IRWIN 1939). If, then, the species-specific properties of Pearlneck serum which differentiate it from Ring dove serum are produced by the action of one or more genes, a segregation of the components particular to Pearlneck, which are present in the species hybrid, is to be anticipated in the offspring of backcrosses to Ring dove. Since various backcross offspring were available, this possibility was put to a test.

The objectives in this experiment were then of two kinds: (a) to test for segregation of properties of the serum peculiar to Pearlneck as compared with Ring dove, and (b) to determine whether such segregating constituents were correlated with the presence of cellular characters also specific to Pearlneck.

Within our knowledge, no definite experimental evidence has previously been presented showing genic effects on the chemical constitution of the serum of any species.

The underlying assumption in all serological studies of the sera of species is that the differences thus shown are most probably due to differences in the proteins. One cannot, however, erase entirely the possibility that other constituents (as carbohydrates or lipoids) may be concerned in the specificities, although in the light of our present knowledge that possibility appears unlikely.

MATERIALS AND METHODS

Antisera were prepared by injecting 0.6 cc of Pearlneck serum (plasma from heparinized blood) intravenously into rabbits three times per week until a minimum of six cc had been injected. The immune sera were collected on the 10th and 11th days after the last injection of serum.

In making the precipitin tests, antiserum was placed in each of a series of capillary tubes, of about two mm diameter, to a height of approximately 6–10 mm, and the serum from the different species or individuals in its serial dilutions was gently layered above. The appearance of a cloudy ring at the antigen-antiserum interface within two hours has been taken

as indicative of a precipitate—namely, a "positive reaction." The same technic was followed with the different "reagents," as explained below. (The precipitate is formed from both antigen and antibody, somewhat the greater proportion coming from the antiserum—that is, the antibody.)

The antibody absorptions were made by mixing undiluted anti-Pearlneck serum with Ring dove serum (the antigen). The amount of antigen in the original absorbing mixture varied from about half to an equal amount of the antiserum. Usually different antisera required different amounts of heterologous antigen to effect a complete exhaustion. The absorbing mixture was stored at 2–5°C for 24 hours, but sometimes it was allowed to remain at room temperature for an hour or two prior to storage in the icebox. If the antiserum was found to be incompletely absorbed, the above process was repeated, small amounts of antigen being added until no ring was formed when the absorbed antiserum, or "reagent," was tested with the antigen as described above.

Extreme care was taken not to overabsorb the antiserum. That is, no more absorbing serum was added to the antiserum than was required just to complete the absorption of antibodies. An excess of the absorbing serum beyond the point at which the absorption of antibodies is complete will produce an inhibition of the precipitin reaction. This may be due to the relative similarity of the proteins of the two kinds of sera being compared. If the sera of two species are distinguished by relatively small chemical differences, an excess of serum from the one added to the antiserum of the other will probably absorb all the antibodies of the antiserum. More comprehensive statements of the chemical relationships of the proteins of species in this respect are given by LANDSTEINER and VAN DER SCHEER (1940).

Individual differences among rabbits in response to injections with serum of Pearlneck, and also of other species, have been commonly observed. Such differences consisted (a) in the variability of different antisera to react with the homologous antigen at high dilutions, and (b) in whether or not the respective antisera would interact with the homologous serum, following absorption with serum of another species. Thus, of 19 different anti-Pearlneck sera, only ten, following respective absorptions by Ring dove serum, produced reasonably strong precipitates with Pearlneck serum, and only four of these were definitely of use in studying segregation of species-specific Pearlneck constituents in the sera of backcross birds. Of the remaining nine antisera, after absorption five reacted only weakly and four not at all with Pearlneck serum.

The (backcross) birds whose sera have been tested in these experiments for the segregation of specific Pearlneck constituents are offspring of matings to Ring dove of the species hybrid and selected backcross hybrids

from the mating of Pearlneck and Ring dove. All the species hybrids from which offspring have been obtained are derived from a single Pearlneck male, thus eliminating the possibility of cytoplasmic influence on the specific Pearlneck constituents in the serum of the species hybrids. The numbers of birds of the species hybrids and successive backcross generations whose sera were individually tested are as follows: five species hybrids, two from the first backcross generation (1/4 Pearlneck, 3/4 Ring dove, or 1/4 P.N.), 12 from the second (1/8 P.N.), six from the third (1/16 P.N.), 13 from the fourth (1/32 P.N.), three from the fifth (1/64 P.N.), seven from the sixth (1/128 P.N.), two from the seventh (1/256 P.N.), and two from the eighth (1/512 P.N.). Likewise the sera from three birds resulting from the backcross to Pearlneck were tested; one of the first backcross generation (3/4 P.N., 1/4 R.D.) and two from the second (7/8 P.N.).

PRELIMINARY CONSIDERATIONS

As previously described by the authors (CUMLEY and IRWIN 1940), the "reagents" by which the species-specific components of the sera of Pearlneck and Ring dove, respectively, are differentiated are prepared by absorbing the antiserum of one by the serum of the other. The relationships of the antigenic components of the sera of these two species and their species hybrids are depicted diagrammatically in figure 1. As will be noted, the serum of each species is portrayed as possessing the greater proportion of its constituents in *common* with the other species and only a relatively small proportion *specific* to itself. In comparison with a similar diagram comparing the cellular components of these same species (IRWIN and COLE 1936a), the proportions of the species-specific components of the respective sera of these species are definitely smaller than those ascribed to the cellular components. Admittedly the proportions as diagrammed for both cellular and serum components are arbitrarily assigned and are to be taken as only relatively representative.

The serum of the species hybrids produced a precipitate, with the specific reagent for Pearlneck serum, at a dilution of antigen somewhat lower than was obtained with Pearlneck serum itself. Thus, Pearlneck serum usually gave a definite precipitate ("ring") with these particular reagents at dilutions of 1:1024 and 1:2048, respectively, while serum from the species hybrids reacted at dilutions varying from 1:32 to 1:256. Nevertheless, serum from the species hybrids by absorption removed all traces of reaction of the respective antisera with the homologous serum. It would be anticipated that any interaction with this reagent of the serum of a backcross bird (to Ring dove) would be at a dilution no greater than that of the species hybrid. Furthermore, if a genetic segregation of antigenic constituents occurred, the reacting capacity of the serum of backcross

individuals, possessing less Pearlneck specific antigen than the species hybrid, might well be correspondingly reduced.

Presumably, the species hybrids contain all or nearly all the species-specific and common components of the sera of both parental species. One would anticipate a segregation of the antigenic complex specific to one species (as Pearlneck) in the offspring of backcrosses of the species hybrids to the other (Ring dove). The underlying assumption is, of course, that the antigens of the serum are genetically determined as are those of the cellular characters.

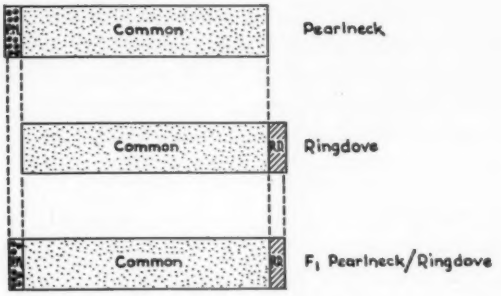

FIGURE 1.—Diagrammatic representation of the antigens of the sera of Pearlneck, Ring dove, and their hybrid.

If the species-specific components of Pearlneck represent but a single antigenic difference from the antigens of Ring dove serum and were genetically determined, the members of the first generation of backcrosses to Ring dove would be of two kinds; those with and those without the character. (Offspring from hybrid males only have been obtained, further reducing the possibility that cytoplasmic influences account for the species-specific properties of Pearlneck in the birds of the various backcross generations.) If two independently inherited characters made up the specific antigenic complex of Pearlneck serum, four kinds of individuals would be expected in approximately equal numbers in the backcross offspring of species hybrids containing these two characters—namely, (a) those with both antigens, (b) those with one, (c) those with the other, and (d) those with neither.

Unfortunately, only two birds of the first backcross generation were available for testing, and the kind of distribution of the antigens in this generation could not be determined. The majority of the birds of the other backcross generations, as given above, represented various cellular characters peculiar to Pearlneck. A few birds possessed no cellular antigens, due to genetic segregation. The sera of all these birds were tested indi-

vidually with each of two reagents for the specific Pearlneck components (provided by the respective absorptions of two anti-Pearlneck sera with Ring dove sera) at serum dilutions of 1:2, 1:4, 1:8, and 1:16. At each test, adequate controls were made of the ability of the specific reagent to react with Pearlneck serum, and not with the absorbing serum (that is, of Ring dove), at each of the above dilutions.

In all the tests, the criterion for the presence of any part of the specific complex was the appearance of a definite precipitate or "ring" at the interface of the serum-reagent mixture, in contrast with no discernible precipitate if Ring dove serum was used. The sera, from the majority of the birds tested, reacted in each of the four dilutions; the highest of these dilutions is given in table 1. Each of these combinations was made twice, and the majority were made repeatedly. Readings on all these tests were made independently by each author. The readings by one were made without knowledge of the contents of the different series of tubes.

EXPERIMENTAL RESULTS

The interactions of the sera of 47 backcross birds, with the two reagents for specific Pearlneck constituents, are given in table 1. Not listed in the table are the reactions of the sera of five species hybrids and three offspring of backcrosses to Pearlnecks (one 3/4 P.N. and two 7/8 P.N.). The serum of one species hybrid did not interact definitely with reagent No. 39S3, for reasons not known to us, although it interacted strongly with the other reagent, No. 198S3. The sera of each of the other four species hybrids and the three offspring of backcrosses to Pearlneck reacted strongly with each of the two reagents.

Both birds of the first backcross generation to Ring dove, D458Y and D458A2, contained at least a part of the antigenic complex specific to Pearlneck, although the serum of D458A2 did not interact with the reagent No. 39S3. The expectation of the kind of distribution in later backcross generations, of individuals with or without a part of the antigenic complex specific to Pearlneck, would depend of course upon the genetic constitution of the backcrossed hybrid parents. Hence in these generations the type of distribution within families is most pertinent. (The offspring from a mating are given a particular number, and the individuals are distinguished by letters—that is, E217C, E217D, and E217J represent individuals of family E217.)

Segregation of serum components specific to Pearlneck

Among the 47 backcross birds, there were 17 whose respective sera reacted definitely with either or both reagents, 24 whose sera produced no definite reactions with either reagent, and the sera of six (D981G, D989L, E40S, E40T, E122P, and E217C) reacted faintly but not definitely with

one or the other reagent. One cannot escape the conclusion that there was a definite segregation of serum constituents among these backcross birds.

Only two individuals whose sera interacted with either reagent had backcross progeny, and their respective offspring were of two kinds—namely, those whose sera reacted with a reagent and those whose sera did not. Thus ♂ D458A2 in two matings to Ring dove produced five birds (D842R2, Z2, E217J, A2, and D2) with a specific Pearlneck component in their sera and four (D842L2, E217D, L2, and B3) without. The serum of one bird of this family, E217C, gave only doubtful reactions with both reagents. Another male, D480D2, in two different matings, had three offspring (E211D, E382C, and E382E) whose respective sera reacted and two (E382F and J) whose sera did not. The progeny of each bird simulated the distribution (50:50) expected if one or more genes on a single chromosome in each backcross parent affected the specific Pearlneck constituents of the serum.

Substantiating evidence of segregation is furnished from the distribution within two other families whose respective backcrossed parents were not alive at the times of these tests. Within the E40 family, the serum of one bird (E40J) reacted definitely, that of each of the three others (E40S, T, and Y) either interacted not all with either reagent or gave only suspicious reactions. For example, the serum of E40T gave no observable precipitate at all in six tests with reagent No. 39S3, and only once in six different tests with reagent No. 198S3 was there even a doubtful reaction at a serum dilution of 1:4. Likewise in the E165 family, the serum of each of three birds (E165N, Z and B2) produced a precipitate with the 198S3 reagent, and that of four others (E165R, V, E2 and F2) did not.

Thus within four backcross families there was a segregation of the birds within each family into two classes—namely, those whose respective sera possessed a serum component specific to Pearlneck and those whose sera did not. These results are explainable only if genic effects on the components of the serum are inferred. However, no information is yet available as to whether the components segregating in the four families are the same or different.

Correlation between components of the serum and blood cells, both specific to Pearlneck

The question naturally arises as to the dependence or independence of the respective antigens specific to Pearlneck in the sera and blood cells of these backcross birds. For example, if a serum component were found only when a particular cellular character also was present, one would need to make tests to determine whether the serum character was detected because of the presence in the serum of products comparable to those of the blood cells.

TABLE 1

Precipitin reactions of the sera of backcross birds with each of two "reagents"—that is, anti-Pearlneck serum absorbed by Ring dove serum.

BACKCROSS PARENT (IF SERUM TESTED)	SERUM FROM BIRDS	PRECIPITIN REACTIONS* WITH EACH "REAGENT"		SPECIFIC PEARLNECK CHARACTERS IN BLOOD CELLS
		39S3	198S3	
	Ring dove	0	0	
	Pearlneck	1024	2048	
	D458Y	4	16+	complex
	D458A2	0	8, 16, 32	d-7
	D480D2	64	64	d-11
	D530F	0	0	d-1
	D771F	0	0	d-5,d-6
	D842L2	0	0	d-7
D458A2	D842R2	32	32	d-7
	D842Z2	0	32	d-7
	D873Y	0	0	none
	D873U2	0	0	none
	D879M	0	4, 8, 16	d-4
	D887C	0	0	d-5
	D965D	0	0	d-2
	D981G	0, ±(4)	0	d-3
	D989L	0, ±(4)	0	d-3
D530F	E16E	0	0	none
D530F	E16G2	0	0	d-1
	E22K	0	0	d-10
	E40J	0, ±(4, 8)	4, 8	d-4
	E40S	0	0, ±(4)	d-4
	E40T	0	0, ±(4)	none
	E40Y	0	0	none
	E54W	0	0	d-5
	E61D	0	0	d-3,d-5
D873Y	E93M	0	0	none
	E122P	0, 8	0	none
	E165N	32	32	d-4
	E165R	0	0	none
	E165V	0	0	d-4
	E165Z	32	16	none
	E165B2	32	32	d-4
	E165E2	0	0	none
	E165F2	0	0	none
D842Z2	E173E2	0	16+	d-7
E61D	E204M	0	—	d-3,d-5
D480D2	E211D	64	32	none
D458A2	E217C	0, ± (16)	0, 32	d-7
D458A2	E217D	0	0	d-7
D458A2	E217J	0	16	†
D458A2	E217A2	0	16	†
D458A2	E217D2	0, 2	16	†
D458A2	E217L2	0	0	d-7

TABLE 1 (*Continued*)

BACKCROSS PARENT (IF SERUM TESTED)	SERUM FROM BIRDS	PRECIPITIN REACTIONS* WITH EACH "REAGENT"		SPECIFIC PEARLNECK CHARACTERS IN BLOOD CELLS
		$39S_3$	$198S_3$	
$D458A_2$	$E217B_3$	o	o	d-7
$D480D_2$	$E382C$	16+	16+	none
$D480D_2$	$E382E$	16+	16+	d-11
$D480D_2$	$E382F$	o	o	d-11
$D480D_2$	$E382J$	o	o	d-11

* The figures given in these two columns represent the highest dilution of serum (antigen) of the respective birds at which a precipitate was noted. "o" represents no precipitate at an antigen dilution of 1:2, nor at higher dilutions; ± indicates a possible but not definite precipitate; 16+ indicates a strong precipitate at the highest dilution of antigen employed, 1:16.

† The cells of each of these three birds contained at the most only a fraction of either or both parts of the d-7 substance.

On the other hand, if there were linkage between the genes for each of two or more cellular characters and those for serum components, qualitative differences would be assumed for the serum components associated with different cellular characters (produced by genes presumably borne on different chromosomes of Pearlneck). But if the genic effects on the serum were not distinguishable, the possibility of duplicate genes on different chromosomes would remain as an alternative explanation.

The fourth column in table 1 shows for each bird whether or not characters particular to Pearlneck were present in its blood cells. Several birds contained no demonstrable character in their cells, as a result of segregation of the causative genes. (It cannot be stated definitely that these birds did not contain any cellular antigens specific to Pearlneck, only that none could be detected by the reagents used at definite concentrations—that is, lower concentrations of antisera might well have revealed other antigens.) Although the numbers possessing the various cellular characters of Pearlneck are too small to permit definite conclusions to be drawn as to correlations between the antigenic characters of the serum with those of the cells, certain relationships seem to exist, as given below.

From the results given in table 1, it can be seen that three birds ($E165Z$, $E211D$, and $E382C$), with no demonstrable cellular antigens specific for Pearlneck, nevertheless possessed in their sera components specific for Pearlneck. There were ten backcross birds ($D873Y$, $D873U_2$, $E16E$, $E40T$, $E40Y$, $E93M$, $E122P$, $E165R$, $E165E_2$, and $E165F_2$) which lacked demonstrable antigens specific to Pearlneck in both cells and serum. Of these, there were two birds ($E40T$ and $E122P$) whose respective sera had given either suspicious or definite reactions with one or the other of the reagents, but duplicate results were not obtained in further tests. These two individ-

uals are therefore classed as having no such antigens in their sera, although the possibility is not excluded that reagents prepared from other antisera (for Pearlneck) might detect antigens specific for Pearlneck in their sera.

Two birds possessing the cellular character d-1 showed no trace of specific Pearlneck antigens in their sera. In addition, no antigens peculiar to Pearlneck were found in the sera of the following birds possessing the respective cellular characters as listed: one (D965D) with d-2, two (D887C and E54W) with d-5, one (E22K) with a part of d-10 (the backcross bird E22K contained in its blood cells only a part of the cellular character d-10, and E40J possessed only a fraction of d-4), two (E61D and 204M) with the combination d-3.d-5, and one (D771F) with both d-5 and d-6. The sera of two birds, D981G and D989L, both carrying the d-3 cellular character, gave faint interactions with the reagent of one antiserum (No. 39S3) but not with the other. The sera of two birds, whose cells contained the d-6 character, were tested with a different reagent than the two given in the table but gave no reaction in repeated trials. Although the sera of birds representing these different cellular characters have not contained antigens specific to Pearlneck, the possibility still exists that the sera of other birds carrying these cellular characters might have such serum components. Also, other antisera may yet be obtained which will provide reagents of different specificities.

Of the six birds (D879M, E40J, E40S, E165N, E165V, and E165B2) whose blood cells contained the cellular character d-4, four (D879M, E40J, E165N, and E165B2) carried in their respective sera a fraction specific to Pearlneck, and two (E40S and E165V) did not. The serum of D879M produced no precipitate in combination with reagent 39S3, whereas a definite ring was formed with reagent 198S3 in each of six tests. Likewise, no more than a suggestive interaction was observed with the first reagent for serum from E40J, but an interaction of its serum at a relatively low titer was produced with the other reagent (No. 198S3). The sera of both E165N and E165B2, however, each carrying the d-4 substance in their cells, interacted definitely with the two reagents. These results suggest the possibility of different antigens in the sera of the two latter birds as compared with that of D879M and E40J, or possibly an additional antigen.

A somewhat similar situation existed for the 12 birds whose blood cells contained one or both parts of the d-7 antigen. (Although the experimental evidence shows that d-7 as originally stated (IRWIN 1939) consisted probably of two distinct substances, it will be considered here as a single character.) Of these, seven (D458A2, D842R2, D842Z2, E173E2, E217J, E217A2, and E217D2) possessed antigens specific to Pearlneck in their respective sera, as indicated by their interactions with the reagent of

198S3, four (D842L2, E217D, E217L2, and E217B3) did not. The serum of one bird (E217C), with each reagent, at one test failed to react and at another gave a positive reaction. The reactions of the sera of these different birds were noted only with the 198S3 reagent. Only the serum of D842R2 interacted definitely with the 39S3 reagent, and this represents the only serious discrepancy of the table. That is, this bird's serum produced a precipitate in combination with the one reagent and that of the parent (D458A2) did not. No rational explanation for this discrepancy can be made. The d-7 cellular character, plus others, was present in the blood cells of D458Y, and the serum of this backcross bird (1/4 P.N.) reacted with both reagents.

Of the four birds whose cells possessed the d-11 character, there were two (D480D2 and E382E) whose respective sera contained antigens specific for Pearlneck and two (E382F and E382J) whose sera did not.

At the present writing there exists no experimental evidence as to whether the one or more specific Pearlneck antigens of the sera of these different birds were the same part or parts of the complex. The differences in reactivity of the two reagents towards sera from the same individuals in some cases suggest that there were qualitatively different components. For example, the reagent from antiserum No. 198S3 interacted strongly with the sera of several birds with which the other reagent produced no (visible) precipitation. Furthermore, qualitatively different antigens, species-specific to the serum of the domesticated pigeon (*Columba livia*) as contrasted with that of Ring dove, have been demonstrated in the serum of two individuals resulting from the backcross of the species hybrids to Ring dove (IRWIN and CUMLEY 1942).

Tests were made on the sera of the offspring of three backcross birds (D530F, D873Y, and E61D), whose respective sera did not contain antigens specific to Pearlneck; and their respective sera also lacked demonstrable Pearlneck antigens. On genetic grounds, of course, no backcross hybrid could contain in its serum a species-specific antigen unless its backcross parent likewise possessed it. The distribution within four families, segregating for both cellular and serum characters, is given in table 2. Families E40 and E165 are segregating for the d-4 cellular character, but their backcross parents were not alive at the time of the tests. D458A2 produced families D842 and E217 in two separate matings to Ring dove; likewise D480D2 produced E211D and family E382 in two matings.

From the summary in table 2, it will be seen that each of the four possible combinations of presence or absence of serum and cellular components are represented in the two families segregating for the d-4 cellular character. The distribution within each family is such that it appears that the one or more genes responsible for the specific Pearlneck antigens in the

blood cells and serum, respectively, are borne on different chromosomes. However, due to the small numbers involved, the possibility of a loose linkage of the causative genes cannot be entirely excluded.

The offspring of D458A2 segregated in approximate equality into those (five) whose respective sera possessed the antigen and those (four) whose

TABLE 2

The distribution of backcross birds within families according to the presence or absence in serum or cells of antigens specific to Pearlneck.

Backcross parent	Cellular character	Cellular antigen + / Serum antigen +	Cellular antigen + / Serum antigen °	Cellular antigen ° / Serum antigen +	Cellular antigen ° / Serum antigen °
Not tested	d-4	E40J	E40S		E40T E40Y
	Number	1	1		2
Not tested	d-4	E165N E165B2	E165V	E165Z	E165R E165E2 E165F2
	Number	2	1	1	3
D458A2	d-7	D842R2 D842Z2 E217J E217A2 E217D2	D842L2 E217D E217L2 E217B3		
	Number	5	4		
D480D2	d-11	E382E	E382F E382J	E211D E382C	
	Number	1	2	2	

The plus (+) sign for the serum antigen represents a definite precipitation with either or both reagents, as given in table 1.

sera did not. (The reaction of the serum of E217C is not included in these figures because of the discrepancies.) If any linkage exists between the gene or genes producing the antigens of the serum and the gene or genes, on each of two chromosomes, whose effects are on the cellular characters, this kind of distribution implies that they were associated with only one of the latter chromosomes.

The segregation in the backcross offspring of D480D2 simulates that expected of an independent segregation of genes producing, respectively, the specific Pearlneck antigens in the serum and blood cells, although no individual was found which lacked antigens in both cells and serum. The

possibility of a loose linkage between the causative genes is not excluded, but seems rather unlikely.

As stated above, no experimental evidence is available as to whether the serum components which are segregating are the same or different. If the serum components in each family are the same, their occurrence in the several backcross hybrids carrying genes for different cellular antigens was only a coincidence. In each backcross family, the distribution (50:50) of the presence or absence of the serum components specific to Pearlneck is typical of that expected if one or more genes on a single chromosome (originally derived from Pearlneck) produce the character. Appropriate tests for qualitative differences are yet to be made on surviving individuals.

DISCUSSION

The evidence presented in this report shows definitely that a segregation of the species-specific components of the serum of Pearlneck, as compared with that of Ring dove, has occurred following backcrosses to Ring dove of species and backcross hybrids. The segregation observed follows exactly that expected if one or more genes on particular Pearlneck chromosomes produced the one or more specific Pearlneck antigens which have segregated as a result of backcrossing.

It is known that one or more genes on at least ten (presumably) different chromosomes of Pearlneck give rise to cellular characters specific to this species, in contrast to Ring dove, No more than a loose linkage, if any, has been found between the gene or genes which produce certain of the different cellular characters (d-4, d-7, and d-11) and those whose effect is on the serum constituents. That is, these tests show that the genes which produce in Pearlneck the antigens of the serum and blood cells, respectively, which distinguish Pearlneck from Ring dove, are different, and as far as can be measured, they are independent in their action on the different antigens.

For the purpose of this discussion, the assumption will be made that the antigens of the serum, as distinguished in these tests, are proteins. It has long been known that individuals of a species differ in the antigenic make-up of their blood cells and that these antigens are gene-determined. The $\bar{A}$ and $\bar{B}$ characters of humans are the best known and the most thoroughly studied. As a result of investigations on the chemical nature of the $\bar{A}$ and $\bar{B}$ antigens, until recently it appeared probable that their specificities are due largely to carbohydrates (see LANDSTEINER 1936 for discussion and references). These findings suggested that the specificities of many cellular characters may well be due at least in part to carbohydrates. Recently, however, LANDSTEINER and HARTE (1940, 1941) presented evidence from studies on the chemical nature of the human A-sub-

stance leading to the proposal that amino acid structures can play a role in the serological specificity of haptenes consisting predominantly of polysaccharides.

In contrast with the known genic effect on blood cells and subsequent segregation of the characters according to Mendelian principles, individual differences in serum proteins are practically, if not entirely, unknown. The few reports which deal with attempts to demonstrate individual differences in the serum proteins of individuals are cited and critically discussed by LANDSTEINER (1936). In this laboratory, no isoprecipitins were detected either in chickens when transfused with chicken blood or, in cooperation with DR. L. C. FERGUSON, in cattle when transfused with cattle blood, although isoagglutinins were readily engendered in each species by these transfusions. From the existing evidence, therefore, one cannot state definitely that genic effects making for differences in the (serum) proteins of individuals within a species do not occur, but at present definite experimental evidence of their existence is lacking. On the other hand, as pointed out by LANDSTEINER (1936), the difference in normal antibodies within a species is presumptive evidence of individual variations in proteins.

Many years ago LOEB (1917) emphasized the importance of a genetic analysis of the differences between the serum proteins of species, stating that "if species-specificity is not a Mendelian character, we are confronted with the possibility that Mendelian mutations may not have been the only essential factor in evolution."

Pursuing the subject still further, LANDSTEINER (1936) states that "As scanty as the knowledge of individual differences of proteins are the data on their inheritance . . . . In order to explain the protein change in the genesis of a new species, one either could imagine in accordance with current theories the occurrence of numerous small variations accompanying single mutations of genes, a hypothesis not yet supported by conclusive proof of hereditary individual protein differences, or one could assume that special mutations cause the transformation, or that a change in the protein constitution takes place only after numerous mutations. If one of the two latter alternatives should prove to be correct, then, in so far as proteins are concerned, a line of demarcation between species would be conceivable."

The segregation of antigens of the serum, specific to one parental species, as found in two different species crosses shows beyond much question that the differences in protein specificity are due to genic action. Thus the point raised by LOEB and cited above is answerable by experimental methods. One might well infer, then, that all the protein constituents are gene-determined. But until differences in serum proteins are found be-

tween individuals, the question as to whether genic effects on serum proteins represent a line of demarcation only between species, and not between individuals, is still unsolved. Neither can it be said whether any one of the segregating antigens of the specific Pearlneck pattern was due to the action of one or of several genes on a single chromosome.

It must be kept in mind that within the serum of a species are to be found different proteins, as globulins, albumins, etc., and one or all of these may have species-specific components in comparison with a comparable protein of another species. In this connection, differences in specificities in egg albumins of various species have been demonstrated by various workers (for leading references, see LANDSTEINER and VAN DER SCHEER 1940). In explanation of the relationships of the proteins of two species, one might assume a minimum of two determinant groupings within each protein, of which one would be peculiar to the species, the other would be similar or identical in both species. An alternative explanation, the two being considered not to be mutually exclusive by LANDSTEINER and VAN DER SCHEER (1940), would be that the proteins are a mixture of closely related components. The arguments for and against each of these explanations are carefully considered by LANDSTEINER and VAN DER SCHEER (1940) in connection with pertinent experimental results.

Whatever may be the eventual explanation for the similarities and differences in proteins of related species, it seems reasonable to conclude that they are gene-determined. This conclusion is made probable by the data given in this paper and also by the finding (IRWIN and CUMLEY 1942) that there are at least three qualitatively different genic effects upon the serum antigens peculiar to pigeon (*Columba livia*) as compared with those of Ring dove. If it be admitted that the species-specific components of proteins are produced by genic action, one can hardly escape the inference that the protein constitution as a whole is likewise affected by genes. But whether the genes act to produce qualitatively distinct determinant groups, or act upon the molecule as a whole, is as yet only a matter of speculation.

SUMMARY

The sera of two dove species, Pearlneck and Ring dove, have been found by immunological technics to have antigenic components *specific* to each and the remainder *common* to the two. The species hybrids contain all, or nearly all, of both *species-specific* and *common* components of both parental species. Following successive backcrosses to Ring doves, a segregation of the antigens *specific* to Pearlneck was noted. In four different backcross families, the segregation of individuals with or without the serum component was that expected from the action of one or more genes on a single chromosome originally derived from the Pearlneck. Since all

backcross individuals are the descendants of a single Pearlneck male, and from species hybrid males only, the possibility of cytoplasmic influence on the serum constituents can be ruled out.

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GENETICS OF PARAMECIUM BURSARIA. III. INHERITANCE OF MATING TYPE, IN CROSSES AND IN CLONAL SELF FERTILIZATIONS[1]

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IN EARLIER papers by the author (JENNINGS 1938, 1939a, 1939b, 1940, 1941a, 1941b) it is shown that in *Paramecium bursaria* there are three groups of stocks the members of which do not conjugate together. These have been designated Varieties I, II, and III (JENNINGS 1941a, 1941b; in earlier papers they were called *groups* I, II, and III). Clones of Variety I may conjugate with other clones of that variety, but do not conjugate with clones of Variety II or III, and corresponding statements may be made for clones of Variety II or Variety III.

Further, in each variety there are definite numbers of mating types which play in the mating behavior the role of different sexes. Variety I and Variety III have each four mating types, while Variety II has eight. Individuals of different mating types within any variety conjugate readily together, but those of the same mating type do not unite in conjugation. All members of a single clone are of the same mating type, except after the infrequent occurrence of clonal self-fertilization, described in the second of these studies (1941b), The single clone is double in its genetic constitution, in such a way that it may by self-differentiation produce a particular two of the four or eight mating types of the variety to which it belongs. Different clones yield in this way different combinations of two types, the particular combination produced being characteristic for the clone. These relations are described in the paper just referred to (1941b).

The present paper is devoted to a study of the inheritance and determination of the mating types, particularly in Variety I with its four mating types; but with some study also of Variety II (eight mating types). A summary of preliminary studies on this matter appeared in the author's papers of 1939b and 1941a.

The main problem involved is the following: At any mating the two parents belong to a particular two of the four or eight mating types of the variety. What are the mating types of the descendants and how are these descendant types related to those of the two parents?

In Variety I matings have been made of each possible combination of two types, and clones descended from 587 pairs of known constitution have

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been examined and their mating types determined. Some of these were produced through self-fertilization of a single clone, others by the crossing of two clones. Since two or more clones were produced from each pair, the number of clones of known parentage that were determined was much greater than the figure just mentioned. In addition clones from 87 pairs of known parentage have been determined as to mating type, in Variety II. Thus the total number of pairs on which the study of inheritance of mating type is based is 674; the total number of clones determined from these pairs was 1146.

METHODS

Many clones collected from diverse localities were kept under cultivation in the laboratory. By testing in the way described earlier (JENNINGS 1941b), the mating type of each clone was determined. Crosses were made by mixing together cultures of two clones of diverse mating type. If the clones are mature, there is as a rule an immediate clotting and mass conjugation between the members of the two different mating types, in the way described in earlier papers (JENNINGS 1939a, 1939b), though in some cases the clotting and conjugation do not occur till the next day or in rare cases still later. The mixtures were usually made in the forenoon. By the late afternoon great numbers of pairs had been formed. Clotting ceases in the evening, but the individuals that have united in pairs remain together. Either in the evening, or the next morning before renewed clotting had begun, single pairs were isolated in the left hand cavity of a depression slide. Here they were allowed to remain till the next day. In the afternoon of that day (36 to 48 hours after union had occurred) the two members of the pair were as a rule separated. One of the two exconjugants was transferred to the right hand cavity of the depression slide, and the two were labeled a (left) and b (right). Each pair was given a number; thus the two exconjugants of pair 12 were designated 12a and 12b.

In most of the crosses—in all the earlier ones—the two exconjugants were allowed to divide, and the two products of the first division were isolated, as a1 and a2, b1, and b2. Division does not occur till 36 to 48 or more hours after separation of the conjugants. If in any case it occurs within 24 hours after separation, it may be suspected that conjugation has not been consummated, and the pair is discarded. Each of the four products of the first fission of the two exconjugants of a pair was allowed to multiply in mass culture, producing a clone, which was designated in the same way as its progenitor. Thus two clones were obtained from each exconjugant, four from each pair. The four clones from each pair are designated a1, a2, b1, and b2, these being employed along with the number given to each pair. Thus from pair 12 there are clones 12a1, 12a2, 12b1,

and 12b2. After the clones had become mature, the mating type of each of the four clones was discovered by testing them in the usual way (JENNINGS 1941b). Some details of the method of testing will be found in a later section of the present paper.

Crosses were also made among the clones descended from the first crosses; and pairs were obtained from the self-fertilization of clones; the procedure just described was followed also with these.

Each clone was cultivated in the lettuce infusion described in the writer's paper of 1939. But with experience certain changes were made in the infusion. It was found that for *Paramecium bursaria* addition of the algal culture to the medium is unnecessary; the Paramecia flourish equally well without it. This simplified much the preparation of the culture medium. The bacterial culture (Chromobacterium) was employed as in the author's previous account. Further, it was found that this lettuce infusion is equally satisfactory when diluted with an equal volume of distilled water, so that this diluted medium was employed in the later cultures.

At first the clones from the exconjugants were kept in small flat dishes known as "caster cups," procurable at the five-and-ten-cent stores. Each was covered with a small plate of glass. But the unexpectedly long period of immaturity, described in the author's paper of 1939a, made these flat dishes unsatisfactory. In later periods the clones were kept in small corked vials or in dishes that were deeper than the "caster cups," and the nutrient medium was renewed at intervals of two or three weeks. It was long before a fully satisfactory method was worked out for keeping the very numerous mass cultures in flourishing condition for many months or for years, and in the meantime many clones were lost that with an ideal method of cultivation might have been saved. To keep the cultures in good condition, about half the culture fluid needs to be drawn off about every ten days and replaced by fresh fluid. At longer intervals it is desirable to transfer a generous sample of the culture to a new clean dish and add fresh fluid. Syracuse dishes are convenient for the clone cultures, but require watching, since at times they unexpectedly lose much fluid by evaporation.

For clones multiplying vegetatively and not recently derived from exconjugants, the culture medium employed was entirely successful without mortality, so long as it was kept reasonably fresh. But many of the clones produced in some of the matings die early, irrespective of the culture fluid or the method of cultivation. Many of the exconjugants die without division. Others divide one or two or a few times, then all the products of fission die. Further, some of the exconjugant clones are weak, so that they can be kept alive and multiplying only by constant care and attention.

DEVELOPMENT OF THE MATING REACTIONS, IN RELATION TO METHODS OF TESTING FOR MATING TYPES

The exconjugant clones produced in the crosses are tested in the following way to discover the mating type to which each belongs. Cultures of clones belonging to each of the mating types of Variety I and Variety II are kept in the laboratory. These cultures are used for testing the new clones; they may be called testers. In testing a new clone culture, a part of the culture, with many individuals, is mixed with each of the tester cultures. This is done on depression slides. Thus a clone culture of Variety I is mixed in different samples with testers of mating types A, B, C, and D. If the clone is mature, it reacts by clotting and forming pairs (as described in JENNINGS 1939a, 1939b) with three of the tester types, not with the fourth. The type with which it does not react is the mating type to which it belongs (since individuals of the same mating type do not react together). The reaction will be spoken of as the sexual reaction.

When the clone is mature, the sexual reactions are entirely definite, so that the mating type to which a given clone belongs is clear. But immediately after conjugation the exconjugant clones are immature; they do not react sexually. This immaturity continues for long periods, from a few weeks (in rare cases), up to many months. During this time of immaturity the clones must be kept in separate mass cultures. In order that this period of laborious culture for the large number of clones shall not be too long, the clones are tested at intervals. At first there is no reaction. After lapse of a period,—often long,—isolated individuals of the clone under test begin to react; they adhere to individuals of the tester clone and eventually form pairs. In clones at the age when reactions begin, the sexual reactions do not occur immediately upon mixture with the tester. Usually there is no reaction on the day the mixture is made. The slides containing the mixtures are kept in moist chambers, often for long periods; they are examined daily for pairs or for adhesions between individuals. After one or two or several days, a pair, or several pairs, may be found in some mixture. Frequently no pairs appear till after a week or more; then perhaps only one or two, or very few. At times a mixture may remain without sign of reaction for several or many days, then a considerable number of pairs may appear. If the isolated pairs that appear are removed, days may pass before additional pairs appear. The appearance of pairs is capricious; if several mixtures with a certain tester are made, isolated pairs may appear in some, not in others.

Why single individuals of a clone thus react sexually while the remainder do not is not clear. The tendency to react sexually appears sporadically even among members of the same clone.

If clones which thus react only in isolated individuals are cultivated

for a sufficient time longer, the tendency to react sexually becomes more widespread among these individuals, until in time all or nearly all react strongly, and pairs are formed *en masse*. In some cases the tendency to strong universal reaction appears rather suddenly—within a few days.

Records show that the scattered reactions which occur when the clone is relatively immature indicate correctly the final mating type of the clone. These isolated reactions occur (in Variety I) with but three of the four tester types. If one or two pairs are formed with each of the testers of types A, B, and D, the clone will be found when mature to belong to the remaining type C, with which no pairs were formed. It is thus possible to discover the mating type of the young clone from these isolated reactions, so that the clone need not be cultivated longer.

An example will illustrate these relations, and also the laborious process of discovering the type of the young clones. In pair 1 of the self fertilized clone 13, the two conjugants were of the mating types A and D. The conjugation occurred May 4, 1938. From the pair six clones were cultivated. Two (a_1 and a_2) came from the first division of one exconjugant, the other four (b_1, b_2, b_3, b_4) from the first two divisions of the other exconjugant. A three-day test of all six with testers A and B beginning June 7 showed no reactions. A nine-day test with A and B beginning June 28 yielded three pairs each with B in clones b_1 and b_3, none in the other exconjugant clones. Beginning July 10, the six clones were tested continuously for fifteen days in mixtures with each of the four types, A, B, C, and D. Clones a_2 and b_1 gave no reactions. Clone a_1 gave on the eighth day a single pair with tester B; b_4 gave after nine days several pairs with tester C; clones b_2 and b_3 several pairs with testers B and C. There were as yet no pairs with mating types A and D. Beginning July 19, all six clones were tested 10 to 15 days (in the different cases) with all four types of testers. During these periods clone a_1 yielded one pair with B and three pairs with C; a_2 gave only one pair, with C; b_1 gave two pairs with B. The clones b_2, b_3, and b_4 gave numerous pairs with B, C, and D, showing that their type was A.

Clones a_1, a_2, and b_1, thus far not determined, were tested beginning September 2 with all four types, for eight to 11 days (in the different cases). During these periods all three gave a few pairs each with B, C, and D, so that all the six clones were now shown to be of type A. It is a curious but not unusual fact that when the clone b_1 was later tested (for thirteen days beginning September 14), it gave no reaction with any of the four types.

In this case therefore tests were kept in progress for at least thirty days for each of the four testers before the mating type of the clones from pair 1 was fully revealed. Successful testing for the mating types of the 1146

clones descended from the 674 pairs of tables 2 and 3 was thus a time-consuming task; it formed the chief occupation of the author and his assistant for two or three years.

Not all the 674 pairs yielded mature living clones for each of the two exconjugants. Some pairs gave four mature clones (two from each exconjugant), some three, some two, some but one. The average (1146 clones from 674 pairs) was slightly less than two clones per pair. It soon became evident that all clones descended from one pair are of the same mating type—in somewhat above 97 percent of the pairs. It was therefore decided to take as the mating type of the clones from any pair the result of tests made on such mature clones as were available from that pair, whether 4, 3, 2, or 1. This may introduce a very slight statistical inaccuracy in determining the proportional number of cases in which from a single pair arise clones of two different mating types—an error certainly of not more than a fraction of one percent.

As illustrated in the case detailed above (pair 1 of self-fertilized clone 13), in some cases there are for some time reactions with only one or two of the four mating types, depending on the chance occurrence of reactive individuals in the mixtures. In such cases if the mixtures are repeated or kept sufficiently long, in time other scattered pairs occur, till they have appeared in the three types to which the young clone does not belong.

In rare cases a phenomenon of different kind appears, one that is of much interest. Members of a particular young clone react persistently with two of the four mating types, not with the other two. Repetition of the tests gives each time the same result—reaction with a particular two only of the four types.

In still other cases reaction occurs readily and in numerous instances with a particular two of the four types, rarely and in isolated instances with a third. In this case the type to which the tested clone is to be assigned is clear; in the former case it is not.

Examples of both these relations were seen in the clones of pair 7 of the mating A×D (clone 13 self-fertilized) (May 4, 1938). There were four clones from this pair, two from each exconjugant; the four are 7a1, a2, b1, and b2.

An eleven-day test, beginning July 10, of each of the four with each of the four types A, B, C, and D yielded one or two pairs with type C in clones 7a1, a2, and b2, none in 7b1. (In the sequel no more pairs were formed with type C.) Next a ten-day test of the four clones with the four types, beginning July 19, gave numerous pairs with types A and B in clone 7a2, one with type A in 7b2, no pairs in 7a1 and b1. Beginning September 2, an eleven-day test of all four clones with the four types gave many pairs with A and B in all four of the clones, none with C or D.

Repeated tests thereafter gave numerous pairs with A and B, none with C or D, in all the four clones. For example, there were in the case of clone 7a2 eleven test periods with each of the four types A, B, C, and D, each period lasting many days. In each of these periods there were numerous pairs formed with types A and B, none with C and D. There were similar tests with the same result in the clones 7a1, b1, and b2.

Thus all clones of pair 7 react readily and in large numbers with types A and B, not with C nor D. Only the early scanty reaction (one or two pairs) of 7a1, a2, and b2 with type C made it possible to identify these clones as belonging to type D. Clones 7b1 reacted with none but A and B.

A few other cases of this sort were observed and thoroughly tested, but such cases are rare. In the clearly demonstrated cases, the reactions occurred with types A and B, none with C and D. In the writer's papers of 1939b and 1941a, this was considered an indication that the two types A and B are closely related and probably differentiate from one and that the same is true of types C and D. The observations thus far are all consistent with this interpretation, but the matter needs further study before this interpretation can be considered established.

THE CROSSES AND THEIR RESULTS

In Variety I the four mating types are A, B, C, and D. Since the members of any mating type may conjugate with members of any of the other types, six different matings are possible; these are A×B, A×C, A×D, B×C, B×D, and C×D. These six matings have been repeatedly made, in some cases between different clones, one of each type; in other cases between the two types resulting from the self-differentiation of a single clone, in which cases the pairs are produced by clonal self-fertilization.

In Variety II the eight types are designated by the letters E, F, G, H, J, K, L, and M. From these eight types there are 28 possible different matings. Only one of these has thus far been investigated, the mating L×M. No study of inheritance has yet been made in Variety III, with its four mating types N, O, P, and Q.

In study and discussion of mating type inheritance, it is important to keep in mind correctly the relation between clones and mating types. Many different clones belong to the same mating type. Usually all members of any clone are of the same mating type, though after the rare occurrence of clonal self-differentiation, the single clone exists in two mating types (details as to this in the second of these studies, 1941b).

In the study of inheritance in Variety I, 22 different parent clones were employed. These (or their ancestors) came from many different localities or were produced in the laboratory from crosses of clones collected in nature. The clones differ much in age or the time they have been in the

TABLE 1

Paramecium bursaria, Variety I, clones employed in study of inheritance, with chief data on each. The table gives: (1) numerical designation of each clone, (2) original designation employed in the laboratory, (3) whether the clone was collected in nature or produced by a cross in the laboratory, (4) date of its collection or origin, (5) locality where collected or parentage, (6) mating type to which it belonged.

1 NUM. DESIG.	2 ORIGINAL DESIG.	3 COLL. OR PROD.	4 DATE OF COLLECTION OR OF ORIGIN	5 LOCALITY OR PARENTAGE	6 MATING TYPE
1	m	Coll.	Apr. 18, 1935	Alexandria, Va.	C
2	l	Coll.	Apr. 18, 1935	Alexandria, Va.	A
4	C	Coll.	Apr. 18, 1935	Alexandria, Va.	C
5	67a1	Prod.	June 18, 1937	1×2	B
8	Lo1	Coll.	March 2, 1938	Baltimore, Md.	D
9	Lo2	Coll.	March 2, 1938	Baltimore, Md.	A
10	Lo3	Coll.	March 2, 1938	Baltimore, Md.	B
11	Lo13	Coll.	March 2, 1938	Baltimore, Md.	D
12	Lo12	Coll.	March 2, 1938	Baltimore, Md.	C
13	He6	Coll.	Apr. 12, 1938	Hebbville, Md.	D
19	J17b1	Prod.	June 7, 1938	8×9	B
21	UC10	Coll.	Feb. 18, 1939	U.C.L.A., Calif.	C
23	3A3	Coll.	Feb. 25, 1939	Three Arches, Calif.	C
24	RHa4	Coll.	March 8, 1939	Rio Hondo, L. A., Calif.	D
27	SGa2	Coll.	March 8, 1939	San Gabriel R., L. A., Calif.	B
32	2H54b	Prod.	Apr. 24, 1939	13, selfed	B
a	2H54b	Prod.	Dec. 1, 1939	32, self-dif.	B
b	2H54b	Prod.	Dec. 1, 1939	32, self-dif.	D
33	2H58a	Prod.	Apr. 24, 1939	13, selfed	B
a	2H58a	Prod.	Nov. 11, 1939	33, self-dif.	B
b	2H58a	Prod.	Nov. 11, 1939	33, self-dif.	A
35	Br48	Coll.	Nov. 8, 1939	Westwood, L. A., Calif.	B
a	Br48	Prod.	Nov. 30, 1939	35, self-dif.	B
b	Br48	Prod.	Nov. 30, 1939	35, self-dif.	C
36	Br48 P1b	Prod.	Nov. 18, 1939	35, selfed	C
38	DM13	Coll.	Dec. 22, 1939	Del Monte, Calif.	D
a	DM13	Prod.	Jan. 31, 1940	38, self-dif.	D
b	DM13	Prod.	Jan. 31, 1940	38, self-dif.	C
39	San29	Coll.	Dec. 23, 1939	Monterey, Calif.	D
44	Br145	Coll.	March 6, 1941	Los Angeles, Calif.	B

Note: "Selfed" signifies self-fertilized. "Self-dif." signifies self-differentiated.

laboratory—the age being reckoned from the time of collection or from the date of the conjugation that produced them. The clones have each been given a number, so far as possible in the order of their age (appearance in the laboratory). These numbers are used in the present paper as the designation of the clones. The more important data as to each of the clones employed in the study of inheritance are given in table 1. Clones of intermediate numbers not given in table 1 were used for other purposes. In

TABLE 2

Paramecium bursaria. Variety I (four mating types A, B, C, D). Mating types of the descendants of pairs from the six possible matings A×B, A×C, A×D, B×C, B×D, and C×D.

	MATING TYPES OF THE DESCENDANTS OF THE DIFFERENT PAIRS, WITH THE NUMBER OF PAIRS THAT YIELD DESCENDANT CLONES OF EACH OF THE FOUR MATING TYPES A, B, C, D				PAIRS IN WHICH THERE WERE CLONES OF TWO MATING TYPES AMONG THE DESCENDANTS		TOTAL NUMBER OF PAIRS
1. A×B							
Clones Mated:							
9×10	31A	39B			4AB	1BC	75
33b×35a	16A	17B	5C	1D			39
Totals	47A	56B	5C	1D	4AB	1BC	114
2. A×C							
9×12	10A	4B	9C	2D	1AD	1BC	27
Miscellaneous	11A	7B	9C	9D	1AB	1BD	38
Totals	21A	11B	18C	11D	1AB, 1AD	1BC, 1BD	65
3. A×D							
9×8	15A	3B	4C	11D			33
13, selfed	15A	1B		13D			29
13, inbred	5A			2D			7
13, inbred	3A	10B		2D	1AB		16
Totals	38A	14B	4C	28D	1AB		85
4. B×C							
1×5		5B	4C				9
10×12		39B		1D	1BD		41
27×23		11B	3C		1BC		15
35, selfed		9B	14C				23
Miscellaneous		3B	1C				4
Totals		67B	22C	1D	1BC	1BD	92
5. B×D							
10×8	3A	8B		1D	1BD		13
35a×32b	24A			25D			49
32, selfed		2B		9D			11
39×44	6A	5B	3C	6D			20
Miscellaneous	2A	4B		4D			10
Totals	35A	19B	3C	45D	1BD		103
6. C×D							
12×8			7C	8D			15
12×11		1B	8C	12D	1BD		22
21×24			3C	16D	2BD		21
38, selfed	1A	2B	13C	15D	1BD		32
36×32b			2C	36D			38
Totals	1A	3B	33C	87D	4BD		128
Grand Totals	142A	170B	85C	173D	17		587

cases where clones denominated a and b are given, these signify the two different mating types produced by self-differentiation of the clone originally bearing the number.

RESULTS OF MATINGS. INHERITANCE OF MATING TYPES

The matings that were made with the mating types of the descendant clones are given in tables 2 and 3.

TABLE 3

Paramecium bursaria, Variety II (eight mating types, E, F, G, H, J, K, L, M). Mating types of the descendants of pairs from the mating of mating type L with mating type M.

L×M; CLONES MATED	MATING TYPES OF THE DESCENDANTS OF THE DIFFERENT PAIRS, WITH THE NUMBER OF PAIRS THAT YIELD DESCENDANTS OF EACH OF THE TYPES	TOTAL PAIRS
Gr14×McD3	12E, 11F, 0G, 8H, 0J, 11K, 21L, 23M	87

General relations and important special features shown by the tables, or supplementary to them, are given in the following sections.

1. The most striking result of the matings, as shown in tables 2 and 3, is the fact (almost but not quite universal) that all the descendants of any one pair are of the same mating type. The two exconjugants of a pair give rise to clones of the same type. This is true in 657 of the 674 pairs shown in the tables—that is, in 97.4 percent of the cases.

As before set forth, four clones were cultivated from most of the pairs, two from each of the exconjugants. All four are finally, as a rule, of the same mating type.

2. The two conjugants of a pair before conjugation are of different mating type. In conjugation they exchange pronuclei, and in consequence the two become of the same type, so that all their descendants are of the same type. It is clear therefore that the mating type depends on the nature of the nucleus formed by the union of the two pronuclei. Further evidence as to this will be found in later sections.

3. The determination of the mating type occurs before the two conjugants have separated, since the two produce clones of the same mating type, notwithstanding the fact that the two are cultivated separately under conditions that are as diverse as are those for members of different pairs which produce different mating types. So far as this observation goes, the determination might be either environmental or genetic. That is, the nature of the nucleus might be affected by the environmental conditions existing at the time of union of the pronuclei. Or the nature of the nuclei might be the resultant of the genetic constitution of the two uniting pronuclei. Further evidence on these possibilities is brought out later.

4. Immediately after conjugation, the clones produced by the exconjugants are immature; they do not react sexually, so that the mating type cannot be determined. Yet the genetic constitution predisposing to a particular mating type is inherited without change in vegetative fission, since the two exconjugants produce clones that when mature are of the same mating type.

5. Different pairs from the same cross (the same two clones and the same two mating types) yield descendants of different mating types. Thus in the mating of type A by type D (table 2), there were 33 pairs from the mating of clone 9 with clone 8, clone 9 being of type A, clone 8 of type D. Fifteen of the 33 pairs produced clones that were of mating type A; three produced clones of type B; four, clones of type C; while 11 produced clones of type D. Other crosses gave similar results.

6. In a few cases there are two mating types represented among the clones descended from a single pair. This was the case in 17 of the 674 pairs of tables 2 and 3—that is, in 2.5 percent of all.

Of the exceptional cases there are two categories. In one category the two members (a and b) of the pair yield clones of different mating type. This was the case in ten out of the 17 exceptional pairs. In the other category are cases in which the two clones descended from a single exconjugant by the first fission after conjugation are of different mating types. This was the case in seven out of the 17 exceptional pairs.

The mating types represented in the cases in which two types are found among the descendants of a single pair may be the same as those of the two parental conjugants or different from them. In the 17 cases in which single pairs gave two mating types among their descendants, these two types were the two parental types in six cases, not the two parental types in eleven cases. At first the author was disposed to exclude cases in which the two parental types occurred in the descendants of a pair, on the ground that this would be the result in case conjugation had in fact not been consummated. But there was in these cases evidence that conjugation had been consummated, since fission did not occur for two or more days after conjugation, and there appears to be no reason to suppose that the exceptional cases would not sometimes consist of the two parental types; in the majority of cases they do not.

In *Paramecium aurelia* the clones derived from the two exconjugants frequently differ in mating type (SONNEBORN 1939); perhaps it may be said that they differ about as frequently as they are alike. The same is true for the two clones derived from the first fission of an exconjugant. The situation that is very rare in *Paramecium bursaria* is thus common in *Paramecium aurelia*.

The underlying cause for the exceptional occurrence of two different

mating types among the clones derived from a single pair in *Paramecium bursaria* is not known. It might be the result of the rare occurrence of self-differentiation in one of the clones, as described in the second of these studies (JENNINGS 1941b). Some cases might be due to the exceptional occurrence of reduction at the third pregamic division.

Since the occurrence of two different mating types in the descendants of a single pair is but a rare exception, which must result from very exceptional conditions, our further discussion will be based on the usual conditions, in which all clones derived from any one pair are of the same mating type.

TABLE 4

Relation of the mating types of the descendant clones to the parental mating types, in the seven classes of crosses of tables 2 and 3. (The 17 pairs not yielding a single type are omitted.)

PARENTAL TYPES (MATING TYPES OF THE CONJUGANTS)	NUMBERS OF PAIRS YIELDING CLONES OF THE PARENTAL MATING TYPES	NUMBERS YIELDING CLONES OF NON-PARENTAL MATING TYPES
A×B	103	6
A×C	39	22
A×D	66	14
B×C	89	1
B×D	64	38
C×D	120	4
L×M	44	43
Totals	525	128

7. In all the crosses the large majority of the descendant clones are of one or the other of the two parental mating types, while a few are of mating types to which neither of the parent conjugants belongs. This important relation is brought out in table 4, based on the data of tables 2 and 3.

Of the 653 pairs represented in table 4, 525 pairs, or 80.1 percent, produce clones that are of the same mating types as one or the other of the two parents, while but 128, or 18.9 percent, of the descendant clones are of types differing from those of the two parents.

In most of the crosses, as shown in tables 2 and 3, the two parental types appear among the descendants in approximately equal number, and each in numbers much greater than any of the non-parental types. This relation is very pronounced in the crosses A×B, A×D, B×C, C×D, and L×M; less marked in A×C and B×D. In the cross B×D there is an exception to this rule; in descendants of this cross the non-parental type A (35 pairs) is more abundant than the parental type B (19 pairs). This exceptional condition results entirely from the single cross of the two clones 35a (type B) and 32b (type D). In this cross, by a remarkable divergence from

the usual rule, none of the parental type B appears among the descendants. Its place is taken by the non-parental type A, which constitutes half of the descendants. The cause for this divergence from the usual situation is unknown.

In some of the crosses the preponderance of the parental types among the descendants is very great. Thus in the cross B×C there are 89 parental types to one non-parental, and in C×D there are 120 parental types to four non-parental. The appearance thus of a single non-parental type among the 89 parental types of the cross B×C is very striking.

8. The two parent conjugants of course represent but two of the four or eight mating types that exist in the variety. The fact that a large majority (about four-fifths) of the descendant clones are of the same type as one or the other of the two parents proves that the determination of the mating type is to a large extent genetic, depending partly on the mating type of the parents.

After conjugation is consummated, any exconjugant consists of a cytoplasmic body that was originally of a certain mating type, and of two united pronuclei, one of the same mating type as the cytoplasmic body, the other of a different mating type.

About half (or somewhat less) of the individuals so constituted take on the mating type of one of the two pronuclei, an equal number (approximately) take on the mating type of the other pronucleus, while the remaining small proportion develop a mating type differing from either of the two pronuclei.

This is well illustrated by the crosses A×B of table 2. In the first cross given in the table (clone 9 by clone 10), each of the 70 pairs included one conjugant that was originally of type A. Follow the fate of these original A's. After completion of conjugation each has a cytoplasmic body that was originally of type A, also one pronucleus that was originally of type A, another of type B. It is to be remembered that both members of the pair (save in rare exceptional cases) yield clones that are of the same mating type. Hence after conjugation about half (31 in this case) of the exconjugants of the constitution just specified become type A, similar to one of the pronuclei, while another half, approximately (39 in this case), become type B, like the other pronucleus. The determination of the mating type thus lies in the pronuclei. In this case, one is dominant in about half the pairs, the other in the remaining half.

In the second A×B cross of table 2 (that of clone 33b by 35a), similar relations appear, except that a small number (six) of the individuals with the A+B pronuclei transform into mating type C (in five cases) or D (in one case).

It is clear therefore that in the large majority of cases at least the deter-

mination of the mating type depends on the nature (as indicated by its origin) of the pronuclei contained by the exconjugant.

9. The fact that the determination of the mating type is partly genetic, depending on the mating type of the parents and hence on the nature of the pronuclei contained by the exconjugants, is further seen in comparing the results of crosses of a clone of particular type with clones of a number of different types. In table 2 we have crosses of clone 9 (type A) with the three clones 10, 12, and 8 of types B, C and D, respectively. Now, keeping in mind the fact that both members of a pair yield clones of the same mating type, the following relations appear. In the cross A×B (or 9×10) about half of the originally A parents (39 out of 70) produce mating type B; in the cross A×C (or 9×12) somewhat less than half of them produce type C, while in the cross A×D (9×8) about half of them produce type D. What the exconjugants that were originally of type A produce depends on what they are mated with.

The only differences between the A exconjugants in the three cases is that they have received pronuclei from three different mating types. This results in transforming approximately half of them into the types from which the pronuclei came.

Similar relations are to be observed if other such comparisons are made. It appears clear therefore that the determination of the mating type depends in approximately half the cases on the nature of the pronucleus that is introduced at conjugation, while in approximately another half it depends on the nature of the pronucleus that is already present.

10. The two exconjugants, originally of different mating types, retain their original cytoplasm; it is only pronuclei that are exchanged. Yet the two produce clones of the same mating type (in more than 97 percent of the cases). It appears therefore that the diversity of cytoplasm does not affect the type produced. It is notable that in the rare cases in which there are two mating types among the descendants of a pair, these are usually not the mating types of the parental cytoplasms (see section 6).

11. The proportion of pairs in which the descendant clones are not of the parental types differs much in different cases. This may be true even in diverse crosses in which the two conjugants are of the same mating type. Thus in the A×B class, in the first cross (clone 9×clone 10) all the descendant clones are of the parental types, while in the second cross (33b ×35a), six out of 39 pairs yield non-parental types. In the A×D crosses, the parental types are as usual much more abundant than the non-parental, except in the fourth cross (inbred clone 13), in which the non-parental type B, with ten pairs, is more numerous than the sum of the two parental types (five pairs). Another still more pronounced case of this

sort is seen in the second of the B×D crosses, in which the non-parental type A entirely replaces the parental type B. In the great majority of crosses the parental types are much more numerous. In some cases the non-parental types are represented only by isolated or nearly isolated pairs. This is the case in A×D (clone 13 self-fertilized), in B×C (cross 10×12), in C×D (cross 12×11), and in 38 self-fertilized. As between matings that are crosses of independent clones and those that are self-fertilizations of a single clone, there is no thoroughgoing difference in this respect.

What underlies the production of a few clones of non-parental types is not evident. Two possible causes suggest themselves:

(1) On the one hand the non-parental types might be the result of recombinations among the genetic factors present in the two clones. Some possible indications of this are seen in the fact that particular crosses produce particular non-parental types and not others, as set forth in section 13, below.

(2) The other possibility is that the non-parental types are produced by certain environmental conditions acting at the time mating type determination occurs—that is, in the two conjugants before they separate. SONNEBORN (1939) finds that the relative numbers of the two mating types produced by pairs in certain varieties of *Paramecium aurelia* depend upon certain environmental conditions. Properly directed experiments on environmental influences are needed for throwing light on this matter in *Paramecium bursaria*.

12. The demonstration that in *Paramecium bursaria* mating type determination is largely genetic makes possible the drawing of another conclusion. The fact that the clones derived from the two exconjugants of a pair are (in the overwhelming majority of cases) of the same mating type indicates strongly that chromosome reduction, so far as it affects mating type, takes place in the first or second pregamic division, not in the third division. For if it occurred regularly at the third division, in many cases the two pronuclei of an individual would be unlike, with the further result that the two exconjugants of many pairs would be unlike in mating type. This matter was elucidated in the author's paper of 1939b, so that a detailed demonstration is not again required. The rare occurrence of cases in which the two exconjugants of a pair yield different mating types might result from the exceptional occurrence of reduction at the third pregamic division of the nucleus.

13. Different matings, so far as the data go, give typically different results as to the mating types produced among the descendants. In Variety I (as shown in table 2) all four mating types are produced among the

descendants of the matings A×B, A×C, A×D, and C×D. In the matings B×C only three types have thus far been produced, there being no descendants of mating type A.

In Variety II (table 3) the mating L×M produces clones of six out of the eight mating types that occur in this variety. Clones of types G and J are lacking among the descendants of the 87 pairs of table 3.

If these same results with the same limitations continue to appear when still larger numbers of pairs are examined in the crosses B×C and L×M, this should upon full analysis throw some light on the genetic constitutions of the different types.

14. To make more complete the genetic picture, the results of self-differentiation and self-fertilization of clones must be added to the facts and relations brought out above. Self-differentiation, self-fertilization, and their results are fully treated in the second of these studies (JENNINGS 1941b). Here will be summarized only the main relations that need consideration in any attempt to discover the genetic mechanism of mating type inheritance. These are as follows:

(1) In any clone derived from an exconjugant, all the individuals belong originally to the same mating type.

(2) At very long intervals self-differentiation occurs in the clone, resulting in the transformation of some of the individuals of the clone into another mating type, so that two mating types are now present, one original, the other derived.

(3) The individuals of the two mating types resulting from self-differentiation now conjugate (self-fertilization of the clone).

(4) The two mating types into which a clone differentiates differ in different cases. Even clones that are originally of the same mating type may produce different derived types. Thus of clones that were originally B, some have been observed to differentiate into B+A, others into B+C, others into B+D. But a given clone when it differentiates always produces the same pair of types, so that the combination of two types which a clone produces is characteristic for the clone. The clone is thus double in its genetic constitution. All the six possible combinations of two from the four mating types of Variety I have been observed: clones that differentiate respectively into A+B, A+C, A+D, B+C, B+D, and C+D. After differentiation, each of the two resultant types may again differentiate into the same two types as before.

(5) By conjugation of the two mating types produced at self-differentiation of a clone ("self-fertilization") additional mating types may be produced. Table 2 includes a number of cases of the production of different types by self-fertilization of a clone.

A clone originally of mating type D has been observed to produce by

self-differentiation, self-fertilization, and later inbreeding among the resulting clones, all the four mating types (A, B, C, D) found in Variety I. A clone originally of type A produced thus the three types A, B, and D. A single clone of type B produced in this way types B and C, while different clones originally of type B have produced thus all the four types A, B, C, and D. Clones of type C are known to yield by self-differentiation and self-fertilization the three types A, B, and C.

GENETIC MECHANISM OF MATING TYPE INHERITANCE

Is it possible from the data set forth above to deduce or suggest a genetic mechanism and its method of operation such as will result in the phenomenon of inheritance described?

Where there are four genetic alternatives, such as the four mating types of Variety I, the suggestion naturally presents itself that the four may depend on two pairs of factors, such as ABab. Eight types, as in Variety II, suggest the operation of three pairs of factors, ABCabc.

But in the detailed working out, no such simple scheme appears satisfactory. In Variety I, for example, the four mating types would be supposed to depend on the two pairs ABab. The most natural assumptions would seem to be that the presence of both A and B produces one of the types; the presence of A only, a second type; of B only, a third type, while the fourth type would result from the absence of both A and B. Thus Type 1 might be either ABab or ABAb or ABaB or ABAB. Type 2 would be Abab or AbAb; type 3 would be aBab or aBaB, while type 4 would be abab.

Investigating the results of the six possible crosses among the four types so represented, it is found that four of the crosses would yield among the descendants all four of the types, while two of the crosses would yield but two types. But in Variety I five of the six possible crosses are known to yield all four of the types, while the sixth ($B \times C$) is already known to yield three of the types. Thus the proposed simple dependence on two pairs of factors will not account for the facts of inheritance in Variety I.

On the whole it appears best to await further knowledge before speculating more on the mechanism of the inheritance.

SUMMARY

When in *Paramecium bursaria* crosses are made between clones of known mating types, the clones that are descended from the exconjugants of these pairs show the following inheritance phenomena.

All descendants of any one pair (of both exconjugants) are of the same mating type. This is true in about 97.4 percent of the pairs; about 2.6 percent are exceptions.

Since the two conjugants are originally of different mating types, but become of the same mating type after exchange of pronuclei in conjugation, the mating type of the descendant clones depends on the nature of the nucleus formed by the union of the two pronuclei.

Since the two exconjugants, though cultivated separately, produce clones of the same mating type, the determination of the mating type occurs before the two conjugants have separated.

The clones produced are for a long time immature, yet the genetic constitution predisposing to a particular mating type is inherited in vegetative fission throughout that period.

Different pairs from the crossing of the same two clones and same two mating types yield descendant clones of different mating types.

In a few cases (about 2.6 percent of the pairs) there are two mating types represented among the clones descended from a single pair. In some such cases the two exconjugants yield clones of different mating type; in others a single exconjugant yields at the first fission two clones of different type. The two mating types may be either those of the two parents or diverse from those of the parents.

In all crosses the large majority of the descendant clones are of one or the other of the two parental mating types, while a few are (as a rule) of other mating types.

Hence mating type determination must be to a large extent genetic; it depends on the mating type of the parents and thus upon the nature of the pronuclei retained by the exconjugant. Nearly half the clones take on the mating type of one of the pronuclei; the remainder take on that of the other pronucleus.

If a certain clone "x" is mated in different crosses with other clones differing in mating type, the clones descended from the exconjugants originally of that clone "x" differ in the different cases. In each case about half of them are of the mating type of the clone with which "x" is crossed. Their mating type depends thus on the nature of the pronucleus that is introduced at conjugation.

Since the two exconjugants, originally of different mating type, retain their original cytoplasm yet produce clones of the same mating type, it appears that the diversity of cytoplasm does not affect the mating type produced.

The proportion of cases in which the descendant clones are not of either parental type differs in different crosses, from 0 up to 50 percent or even more in rare cases.

The fact that the clones derived from the two exconjugants are of the same mating type indicates that chromosome reduction, so far as it affects mating type, occurs in the first or second pregamic nuclear division, not

in the third. The rare occurrence of cases in which the clones from the two exconjugants differ in type may be due to the exceptional occurrence of reduction at the third division.

In Variety I, five of the six possible crosses among the four mating types yield all four mating types among their descendants; these are the matings A×B, A×C, A×D, B×D, and C×D. The sixth cross, B×C, has thus far been observed to produce only the three mating types B, C, and D. It appears possible that more extensive study may yet show this cross to produce also the remaining type A. In Variety II the mating L×M has yielded six of the eight types of this variety—namely, E, F, H, K, L, M.

In self-differentiation and self-fertilization of clones, part of the members of the clone, originally all of one type, transform into a second type. The two types then conjugate. Different clones, even those originally of the same type, differentiate into different pairs of types. The combination of two types thus produced by any clone is characteristic for the clone. The single clone is thus double in its genetic constitution. All possible combinations of two types out of the four of Variety I have been observed: A+B, A+C, A+D, B+C, B+D, C+D.

By conjugation of the two types produced at self-differentiation, additional mating types are produced. Thus from a clone originally of a single mating type, several or all of the four mating types (Variety I) may be produced. A clone of D type has produced all four types; one of A type has produced A, B, and D; one of C type, A, B, and C; one of B type, B and C.

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AN ANALYSIS OF DATA ON X-RAY-INDUCED VISIBLE GENE MUTATIONS IN DROSOPHILA MELANOGASTER[1]

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INTRODUCTION

IT IS well known that the chromatin material of germ cells is subject to spontaneous alterations. These changes may involve large sections of the chromosomes, or they may be localized about a particular gene. Since these alterations are subject to variations, both random and non-random in origin, the methodology by which this variation is analyzed becomes of major importance to any interpretation to be attached to such data.

Previous irradiation experiments have shown that changes in the chromatin can be produced artificially and at a higher frequency than exists naturally. Differing sources and dosages of radiant energy have been used, and differing types of gene changes have been studied. All those experiments likewise involve, as an integral part of their interpretation, the random and the non-random variation mentioned above, and its proper analysis.

If we have a population of Drosophila in which a certain fraction, p, has mutated from the normal red eye-color to a vermilion eye-color, we say that the probability of observing a mutant fly, on one random observation of this whole population, is p. If the proportion of mutants stays fixed during the period of our interest in the population, it is well known that the probability of observing exactly r mutants in a sample of size n is given by the binomial probability function

$$(1) \qquad \frac{n!}{(n-r)!\,r!}\,p^r(1-p)^{n-r}.$$

The population is determined by p, which, however, is usually unknown. Physical or biological considerations supply certain hypotheses which are then used in the estimation of p. We then have a means of determining what we would expect to obtain during random sampling from the given population. If expectation and observation fail to agree, doubt is cast upon the adequacy of the hypotheses adopted.

For the purpose of measuring the agreement between observation and expectation, it has been thought advantageous to approximate the discontinuous function, (1), by a continuous function. The Normal distribu-

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tion has been used for that purpose, but has not been shown to be a good approximation if the expected numbers, np, are small. The probabilities associated with visible gene mutations are nearly always so small that n would need to be much larger than is experimentally possible before the Normal approximation would be—in the light of present knowledge—a safe one to use in the analysis of such irradiation data. In spite of this situation, the literature on gene mutations shows that the Normal approximation has been used in the classical large sample manner to test the variability observed in irradiation data. It seems that the error involved in such analyses should be investigated or else a different method of analysis sought.

This paper has the dual purpose of presenting some new data on X-ray-induced, visible gene mutations and of showing how a satisfactory analysis of such data can be made by means of the χ^2 distribution.

HISTORICAL BACKGROUND

Due to the difficulties attending the collection of data on visible gene mutations, there is not an abundance of such data in the literature. Such data as are found are characterized by large numbers of observations and small numbers of recorded mutations. If the data are analyzed at all, the analysis is accomplished through the Normal approximation described above and upon the basis of large sample theory. These circumstances leave in doubt the conclusions drawn from the data.

The χ^2 test is especially designed for enumeration data. Its purpose is to measure the agreement between the numbers actually observed and the corresponding numbers which would be expected mathematically upon the basis of certain hypotheses. Thus this test seems particularly adapted to the general nature of irradiation research on visible gene mutations. The χ^2 test has two other advantages: (1) it has a form especially adapted to convenient calculation of $2 \times n$ classifications, and (2) its additive property enables one to accumulate evidence on rare events statistically.

However, one big disadvantage has been thought to attach to the use of χ^2 when small expected numbers are involved. Since an exact test is not practical on most data, it has been approximated by means of a Type III Pearsonian curve. This distribution is the one found in the tables. It was thought, until quite recently, that this approximation was so poor, when small numbers such as those which exist in irradiation data are used, that it could not validly be employed.

The closeness of the Type III approximation, in so far as it affects a test of significance, has been investigated by HOEL (1938), NEYMAN and PEARSON (1931), SUKHATME (1938), COCHRAN (1936), and FRYER (1940). These investigators have shown that the tabular χ^2 distribution is satis-

factory for a test of significance even when the expected numbers are as small as 0.5. This closeness of agreement between the exact and the tabular distributions of χ^2 has been demonstrated only empirically; but the validity of that demonstration has no relation to the assumptions accompanying the derivation of the tabular distribution. However the tabular values may have been obtained, they seem to give the exact distribution of χ^2 very satisfactorily. These considerations plus the additive property of χ^2, which makes it possible to collect information on rare events, make it appear that the χ^2 test fits the purposes of irradiation research quite well.

THE EXPERIMENT

Materials and methods

The experiments involved three wave lengths of radiation, two dosages with each wave length, and 19 specific gene loci.

The following stocks of females of *Drosophila melanogaster* were maintained:

(1) $\dfrac{sc\ ec\ cv\ v\ f\ car}{sc \qquad v\ ClB}\ \dfrac{+}{+}\ \dfrac{+}{+}\ \dfrac{+}{+}$;

(2) $\dfrac{+}{+}\ \dfrac{al\ dp\ pr\ c\ px\ sp}{al\ \ Cy\ \ L_4\ \ sp}\ \dfrac{ru\ h\ st\ sr\ e^s\ ca}{Me'\ \ Sbc\ \ e^s}\ \dfrac{+}{+}$;

(3) $\dfrac{+}{+}\ \dfrac{+}{+}\ \dfrac{ru\ h\ st\ sr\ e^s\ ca}{ru\ h\ st\ sr\ e^s\ ca}\ \dfrac{+}{+}$;

(4) $\dfrac{+}{+}\ \dfrac{+}{+}\ \dfrac{ru\ h\ st\ sr\ e^s\ ca}{Me'\ \ Sbc\ \ e^s}\ \dfrac{+}{+}$; and,

(5) $\dfrac{+}{+}\ \dfrac{al\ dp\ pr\ c\ px\ sp}{al\ \ Cy\ \ L_4\ \ sp}\ \dfrac{+}{+}\ \dfrac{+}{+}$.

Normal males from a line which had been inbred for several generations were X-rayed at 24 hours, or less, of age. These Drosophila were irradiated with a gas-type X-ray tube of WYCKOFF and LAGSDIN'S (1930) general design, a description of which has been presented by PINNEY (1939). The targets were the pure metals: silver, copper, and chromium. The particular X-rays were filtered heavily by a window of palladium for the silver target, nickel for the copper target, and aluminum for the chromium target. The thickness of this window was adjusted to absorb fifty percent of the radiation.

The peak kilovoltage across the tube was different for the different metals—56.0 for Ag, 39.6 for Cu, and 34.3 for Cr—as measured by a 10 cm sphere gap. The current through the tube during the irradiations was held constantly at 12.5 milliamperes. The beam intensity at the time of irradiation was measured either by a small ionization chamber or by a

Victoreen dosimeter. The ionization chamber was constructed in the IOWA STATE COLLEGE machine shop after a design by L. E. PINNEY based upon the general plan of TAYLOR and SINGER (1930). The dosimeter was checked frequently with the ionization chamber both for accuracy and for the absorption of the particular rays in the capsule housing the test chamber.

The ionization readings for the different metals were corrected for a temperature of 20°C, a barometer reading of 74 cm, and the loss of radiation due to the amount absorbed in the 5.5 cm layer of air between the surface of irradiation and the volume giving the ionizing current.

The linear absorption coefficient for the male Drosophila was determined for each wave length used. The sperm-storage organs are found throughout the abdomen, but for the purposes of X-ray dosage, average approximately the mid-position. The energy incident upon the sperm is also corrected for this loss of X-ray as they pass into the body of the fly. These corrected readings are those presented in the tables.

It is essentially true that for a constant thickness of biological material the fraction of the incident dosage absorbed by the material is a constant for a given wave length of irradiation. Hence $\log I/I_0 = -\mu x$, where I_0 is the incident dosage. The ratio: I/I_0, was obtained experimentally as follows: a fly was placed directly over the window of the ionization chamber and then irradiated. The dosage incident upon the fly and that passing through the fly were both measured. When the latter had been expressed as a fraction of the former, and the process repeated on several flies, the average was used as the estimate of I/I_0. This gave $I/I_0 = 0.53$, 0.894, and 0.246 for Cu, Ag, and Cr, respectively.

These estimates were then used to plot $\log I/I_0$ against an arbitrary depth scale. From these graphs, it was estimated that 94.4, 72.5, and 49.5 percent of the dosage incident upon the fly was actually incident upon the sperm in the mid-position for Ag, Cu, and Cr irradiation, respectively.

The average effective wave length of the X-rays for the different metals, under the conditions of this investigation, was determined by absorption experiments through successive sheets of aluminum, 0.1308 mm and 0.0072 mm in thickness. These tests showed the average effective wave lengths of irradiation to be as follows: Ag, 0.7Å, Cu, 1.5Å, and Cr, 2.1 to 2.2Å.

Male Drosophila which had been X-rayed under the above conditions were then mated to virgin females from one of the stocks described. Whenever a mutation was believed found, that fly was mated back to an appropriate stock. Due to the high percentage of sterility found with the high dosages used, those progeny tests frequently produce no offspring. Those mutations which were verified by progeny tests appear under the heading "v"; the rest are classified as "c" if the identification was considered fairly certain.

Under fortunate circumstances, a progeny test also furnishes a test for a deficiency. Since at these dosages, many of our mutations are not classifiable as "deficient" or "non-deficient," that distinction has not been made in the data to be presented below.

Results

The data obtained in the above-described experiment are presented in the tables below. In tables 2, 3, and 4, only those sets of data which are

TABLE 1

Summary of data on visible gene mutations by wave length, dosage, and chromosome.

TARGET	DOSAGE (r units)	I LOCI EXAMINED	I v	I (v+c)	II LOCI EXAMINED	II v	II (v+c)	III LOCI EXAMINED	III v	III (v+c)
Cu	3,625	52,101	16	32	18,850	4	8	13,917	3	7
Cu	7,250	12,455	12	23	3,432	0	0	5,451	2	5
Cr	2,475	7,020	0	1	8,331	2	7	7,478	1	4
Cr	4,950	6,397	2	8	7,576	1	5	6,760	3	4
Ag	3,776	—	—	—	4,412	0	3	4,389	0	5
Ag	4,720	2,955	2	4	3,416	0	2	4,005	1	5
		80,928	32	68	46,017	7	25	41,970	10	30

TABLE 2

Summary of specific mutations on Chromosome I.

LOCUS	Cu_{3625} LOCI EXAMINED	Cu_{3625} v	Cu_{3625} (v+c)	Cu_{7250} LOCI EXAMINED	Cu_{7250} v	Cu_{7250} (v+c)	Cr_{4950} LOCI EXAMINED	Cr_{4950} (v+c)
sc	7,443	3	4	1,765	2	2	667	0
ec	7,443	3	8	1,765	2	5	667	2
cv	7,443	1	2	1,765	0	0	667	0
ct	7,443	4	8	1,765	5	9	667	5
v	7,443	3	6	1,765	1	2	667	1
f	7,443	1	2	1,765	1	2	667	0
car	7,443	1	2	1,765	1	3	667	0
	52,101	16	32	12,455	12	23	4,669	8

sufficiently complete to be useful are included. As with most irradiation data on visible gene mutations, the scarcity of these data limits the definiteness of any conclusions to be drawn from this one set of data. Fortunately, the χ^2 test enables one to add this evidence to that obtained in other experiments testing the same hypotheses.

TABLE 3

Summary of specific mutations on Chromosome II.

LOCUS	Cr_{2475} LOCI EXAMINED	(v+c)	Cr_{4950} LOCI EXAMINED	(v+c)	Cu_{3625} LOCI EXAMINED	v	(v+c)
al	1,843	2	1,638	0	3,483	1	1
dp	929	1	860	1	2,971	1	4
pr	929	2	860	1	2,971	0	0
c	929	2	860	2	2,971	0	0
px	929	0	860	1	2,971	2	3
sp	1.843	1	1,638	0	3,483	0	0
	7.402	8	6,716	5	18,850	4	8

TABLE 4

Summary of specific mutations on Chromosome III.

LOCUS	Cu_{3625} LOCI EXAMINED	v	(v+c)	Cu_{7250} LOCI EXAMINED	(v+c)	Cr_{4950} LOCI EXAMINED	v	(v+c)
rh	2,222	0	1	884	2	787	0	0
h	2,222	0	0	884	1	787	0	0
st	2,222	1	2	884	0	787	1	2
sr	2,222	1	1	884	0	787	0	0
e^s	2,807	1	2	1,031	0	1,638	2	2
ca	2,222	0	1	884	2	787	0	0
	13,917	3	7	5,451	5	5,573	3	4

ANALYSIS OF THE DATA

The above data furnish some evidence regarding questions of interest in irradiation research. Some of these questions are: (1) Are there differential rates of mutation at the gene loci considered? (2) Is the mutation rate of a particular gene directly proportional to the dosage of radiation applied? (3) After due allowance is made for differences in absorption, does wave length affect mutation rate? (4) In so far as the sets of genes observed are representative of their respective chromosomes, what evidence is there of different rates of mutation among the first three chromosomes of *Drosophila melanogaster?*

To obtain evidence on these questions, we shall derive from them certain hypotheses, which then become the "base-lines" from which variation in the data is measured. If the deviations from those "base-lines" are too great to be reasonably assigned to random sampling variation, we shall

conclude that the hypotheses used probably are not taking into account all the sources of non-random variation.

The χ^2 test is used to distinguish between the random and non-random variation described above.

The first question stated above leads to this null-hypothesis: for a given wave length, the probability of observing a mutation on one observation of a given gene locus is the same for all the loci considered in that set. The expected numbers under that hypothesis are compared with the corresponding observed numbers in table 5. The calculation of the expected numbers is illustrated from table 3 and for Cr_{2475}.

Let p be the estimated probability of observing a mutation at a particular locus on a single observation. Then we would expect (mathematically) 1843 (p) mutations at "*al*" and "*sp*" and 929 (p) mutations at each of the other loci listed. Hence, keeping the total number of mutations expected the same as the number observed, we must have $2(1843p)+4(929p)=8$. This requires that $p=.001081$; or, the estimated rate of mutation for each locus is 1081 per million observations. One then obtains

$$\bar{x}=1843(.001081)=2.0$$

as the expected number of mutations at the locus "*al*."

As pointed out by FRYER (1940), we can use

$$\chi^2=\sum_{1}^{N}\frac{(x_i-\bar{x}_i)^2}{\bar{x}_i}$$

and disregard the numbers of non-mutants, since the deviations squared are very small compared to the expected numbers of non-mutants.

The tests described above were made on the data of tables 2, 3, and 4. The results for each test separately are given in table 5. χ^2 offers a means

TABLE 5

Tests of the hypothesis that basic rates of mutations of genes within sets are the same.

METAL AND DOSAGE		CHROMOSOME I χ^2	I d. f.	I P	II χ^2	II d. f.	II P	III χ^2	III d. f.	III P
Cu_{2825}	v	4.10	6	.66	5.29	5	.40	2.82	5	.77
	v+c	9.94	6	.12	12.52	5	.035	2.14	5	.83
Cu_{7250}	v	9.08	6	.18	—	—	—	—	—	—
	v+c	15.58	6	.023	—	—	—	6.25	5	.28
Cr_{2475}	v+c	—	—	—	3.50	5	.63	—	—	—
Cr_{4850}	v	—	—	—	—	—	—	3.82	5	.57
	v+c	18.30	6	.007	5.94	5	.31	6.51	5	.27

of combining the results of separate tests if that is desired. A pooling of the data into one test occasionally yields misleading results. For example, the pooled χ^2 for the first chromosome has six degrees of freedom and is far beyond the 0.1 percent level of significance; but during the copper irradiation—where we obtained our best set of data—the χ^2 is well above the five percent level of significance. Also, on the second chromosome, the pooled χ^2 is definitely non-significant. This hides the fact that χ^2 was significant for the results of the copper irradiation.

One sees from table 5 that but three of the fourteen values of χ^2 are significant and but one is highly so. All three of the significant values came on the (v+c) data. It is found from the amounts contributed to χ^2 by the various genes that the "*ct*" locus is causing the large χ^2 in two of the three significant cases. For example, for Cr_{4950} on I, the expected number of mutations at each locus was 1.14. Actually five mutations were observed at "*ct*." Then $(5-1.14)^2/1.14=13.07$ of the 18.30 found for the χ^2 in this case was produced by the deviation of the observed number of "*ct*" mutations from the number of such mutations expected if all of the sex-linked genes considered have the same mutation rate.

In the remaining case, "*dp*" and "*px*"—both more subject to misclassification than most of the genes observed—are contributing most of the deviations from expectation. Assuming that neither wave length nor dosage of irradiation interact with mutation rate, it is proper to use a combined χ^2 test on the data of table 5. Adding the values of χ^2 for "v" mutations, if possible, one obtains: $\chi^2=59.10$, d. f. $=48$, $P=.05$. If this same procedure is used on "v" mutations only, $\chi^2=25.11$, d. f. $=27$, $P=.57$. Hence one concludes that, in general, the genes in a chromosome group have about the same mutation rate. However, there is evidence in our data to indicate that the rates of change at the "*ct*" locus might be greater than the rates of mutation of the other genes in the corresponding chromosome set.

The data in table 1 furnish evidence regarding the proportionality of mutation rate to dosage. If one makes the comparisons: Cu_{3625} with Cu_{7250} on the first and third chromosomes, and Cr_{2475} with Cr_{4950} on the second and third chromosomes by assuming that mutation rate is proportional to dosage applied, one obtains these results in that order, using "*v*" mutations:

$\chi^2=1.37$, d. f. $=1$, $P=.25$,
$\chi^2=0.032$, d. f. $=1$, $P=.85$,
$\chi^2=1.16$, d. f. $=1$, $P=.29$, and
$\chi^2=0.176$, d. f. $=1$, $P=.68$.

Since none of the χ^2 values above is significant, it is concluded that the hypothesis of proportionality of mutation rate to dosage is in accord with the data in table 1.

In studying the effect of wave length on mutation rate by means of χ^2, we shall assume that wave length—for a given dosage and gene locus—has no effect on mutation rate. That is the hypothesis to be tested against the observed data. Since previous analyses cast no doubt on the hypothesis that mutation rate and dosage of radiation are proportional, we have assumed that to be true in the analyses below. Using the appropriate parts of table 1, we have derived the contents of tables 6 and 7.

TABLE 6

Comparison of wave-lengths using (v) frequencies and sex-linked mutations.

WAVE LENGTH	(1) DOSAGE (r units)	(2) LOCI EXAMINED	(1)×(2) TOTAL	MUTATIONS OBS.	MUTATIONS EXP.
0.7Å (Ag)	4,720	2,955	.04295	2	1.4
1.5Å (Cu)	3,625	52,101	.5815	16	18.6
	7,250	12,455	.2780	12	8.9
2.1 to 2.2Å (Cr)	4,950	6,397	.09755	2	3.1
Total (1)×(2)=	324,777,625		1.00000	32	32.0
	χ^2=2.09,	d. f.=3,	P=.56.		

The results in table 6 indicate that the hypotheses adopted account for the variation among the data quite well.

TABLE 7

Comparison of wave lengths using (v) frequencies and third chromosome mutations.

WAVE LENGTH	(1) DOSAGE (r units)	(2) LOCI EXAMINED	(1)×(2) TOTAL	MUTATIONS OBS.	MUTATIONS EXP.
0.7Å (Ag)	4,720	4,005	.1175	1	1.2
1.5Å (Cu)	3,625	13,917	.3137	3	3.1
	7,250	5,451	.2457	2	2.5
2.1 to 2.2Å (Cr)	2,475	7,478	.1151	1	1.1
	4,950	6,760	.2080	3	2.1
Total (1)×(2)=	160,842,525		1.0000	10	10.0
	χ^2=0.531,	d. f.=4,	P=.96.		

While the agreement between observation and expectation is abnormally good for these data, it is not excessively so. In consideration of previous analyses, this result seems to confirm that in table 6.

A comparison of the rates of mutation on the three chromosomes studied can be made if one makes two assumptions in addition to the null hypothesis that the three chromosomes have the same basic rate of mutation.

These assumptions are: (1) that the sets of genes we have observed are equally representative of their respective chromosomes, and (2) that differences among the stocks of flies used do not spoil the comparisons being made.

In view of the results of previous analyses, we may use all the data in table 1 in an analysis similar to that used in tables 6 and 7. This is done in table 8.

TABLE 8

Comparison of mutation rates on the first three chromosomes of Drosophila melanogaster.

CHROMOSOME	"UNITS"	EXPECTED PROPORTION OF MUTATIONS	MUTATIONS	
			OBS.	EXP.
I	342,152,125	.4862	32	23.8
II	184,116,907	.2616	7	12.8
III	177,415,389	.2521	10	12.4
	703,684,421	1.0000	49	49.0

$\chi^2 = 5.918$, d. f. = 2, P = .056.

Although the probability, P, in table 8 is not quite down to the conventional five percent level, one must conclude that the variation from expectation probably is not just random. We cannot say whether the nonrandom element is produced by stock differences, or by different mutation rates on the chromosomes.

ANALYSES OF ALLIED DATA

The literature contains a number of sets of data which are closely related to this study. A few of these sets have been analyzed in order to illustrate the kind of information they can yield. The data selected for analysis pertain to these subjects: (1) continuous versus interrupted administration of the dosage of radiation; (2) the effectiveness of heat in producing sex-linked lethals in Drosophila; (3) the effectiveness of heat in producing visible gene mutations; (4) differential mutation rates among different species of Drosophila; (5) rates of reverse mutation; that is, from recessive to normal, and (6) mutation rates of the alleles in "white" series of *Drosophila melanogaster*.

The data for the first illustration are from PATTERSON (1931) and are presented in table 9.

The only one of the above experiments to yield a significant χ^2 was the second. The author expressed the feeling that since it was the smallest scaled and for the shortest total time, it was the least reliable. Accepting that point of view and noting the total χ^2, the general conclusion to be

TABLE 9

Continuous versus interrupted administration of the radiation as it affects rate of lethal mutation in Drosophila.

TIME INTERVAL OF INTERRUPTION	TOTAL TIME	DOSAGE (r units)	NUMBER OF FLIES	NUMBER OF LETHALS OBS.	NUMBER OF LETHALS EXP.	NUMBER OF NON-LETHALS OBS.	NUMBER OF NON-LETHALS EXP.
0 hrs.	16 mins.	1,654	971	49	60	922	911
12 hrs.	16 mins.	1,654	993	62	61	931	932
6 hrs.	16 mins.	1,654	981	71	61	910	920
		$\chi_1^2=3.92$, d. f.$=2$, P$=.15$					
0 hrs.	8 mins.	2,558	518	39	50	479	468
12 hrs.	8 mins.	2,558	345	45	34	300	311
		$\chi_2^2=6.63$, d. f.$=1$, P$=.01$					
0 hrs.	10 mins.	1,234	863	28	31	835	832
24 hrs.	10 mins.	1,220	876	31	32	845	844
12 hrs.	10 mins.	1,221	936	40	34	896	902
8 hrs.	10 mins.	1,219	856	34	31	822	825
1 hrs.	10 mins.	1,220	1,014	32	36	982	978
30 mins.	10 mins.	1,234	962	33	35	929	927
		$\chi_3^2=2.31$, d. f.$=5$, P$=.80$					
0	12 hrs.	radium	544	58	57.9	486	486.1
12	12 hrs.	radium	452	48	48.1	404	403.9
		$\chi_4^2=.000425$, d. f.$=1$, P$=.98$					
		$\sum_1^5 \chi_i^2=12.86$, d. f.$=8$, P$=.12$					

TABLE 10

*Summary of an investigation of the production of sex-linked mutations by temperature shocks.**

TYPE OF INVESTIGATION	TREATMENT	GAMETES EXAMINED	SEX-LINKED LETHALS OBS.	SEX-LINKED LETHALS EXP.
Lethals	Controls	6,495	10	15.7
(using *ClB* Method)	♂, 35°–38°C	4,635	19	11.2
	♀, 35°–38°C	7,052	15	17.1
		18,182	44	44.0
	$\chi^2=7.76$, d. f.$=2$, P$=.02$			
			VISIBLE MUTATIONS	
Visible mutations	Controls	84,015	8	12.8
(with attached-X stocks)	♂, 35°–38°C	88,198	17	13.4
	♀, 35°–38°C	64,300	11	9.8
		236,513	36	36.0
	$\chi^2=2.91$, d. f.$=2$, P$=.24$			

* See BUCHMANN and TIMOFEÉFF-RESSOVSKY 1936.

drawn from the above data would be that interruption of the process of irradiation probably does not affect the production of lethal mutations.

On the subject of heat-induced mutations, BUCHMANN and TIMOFEËFF-RESSOVSKY (1936) have reported an investigation during which they produced sex-linked lethals in one stock of Drosophila and visible gene mutations in an attached-X stock of Drosophila. They used both X-rays and heat, but only the data from the latter treatments are given here.

TABLE 11

Comparison of rates of lethal, sex-linked mutation in D. funebris and D. melanogaster (from TIMOFEËFF-RESSOVSKY *1937).*

SPECIES	TREATMENT	NUMBER EXAMINED	LETHALS		NON-LETHALS	
			OBS.	EXP.	OBS.	EXP.
funebris	controls	2,869	2	1.8	2,867	2,867.2
	X-rayed	1,037	84	96.8	953	940.2
melanogaster	controls	1,837	1	1.2	1,836	1,835.8
	X-rayed	731	81	68.2	650	662.8
		$\chi^2=4.57$, d. f. = 2, P = .11				

TABLE 12

Comparison of rates of lethal sex-linked mutation in D. simulans and D. melanogaster (from KOSSIKOV *1935).*

SPECIES	TREATMENT	NUMBER EXAMINED	LETHALS		NON-LETHALS	
			OBS.	EXP.	OBS.	EXP.
simulans	Controls	1,446	12	10.6	1,434	1,435.4
	X-rayed	842	42	38.0	800	804.0
melanogaster	controls	469	2	3.4	467	465.6
	X-rayed	1,019	42	46.0	977	973.0
		$\chi^2=1.57$, d. f. = 2, P = .47				

It appears from table 10 that the variability between control ♂ and ♀ could very well be random as far as the visible mutations are concerned. As regards the sex-linked lethals, it seems logical to conclude that heat treatments probably did increase their rate of mutation somewhat.

The third type of comparison we shall make concerns different species of Drosophila. The rates of mutation of these species are compared in tables 11 and 12, using lethal mutations for the comparison.

It is seen from tables 11 and 12 that the variability exhibited by those sets of data as wholes (which is the way the experiments were planned)

may reasonably be supposed to be random variability rather than being due to species differences in mutation rate. However, it is of interest to note that if in table 11 one omits the controls from consideration, one ob-

TABLE 13

Rates of mutation from visible recessive to normal.

LOCUS	(A) DATA FROM TIMOFEËFF-RESSOVSKY 1937*			(B) DATA FROM JOHNSON AND WINCHESTER 1934 3975 r.		
	NUMBER OF GAMETES	MUTATIONS		NUMBER OF GAMETES	MUTATIONS	
		OBS.	EXP.		OBS.	EXP.
y	21,897	0	1.9	69,923	1	2.9
sc	17,676	3	1.6	101,042	5	4.3
w	29,233	2	2.6	—	—	—
w^a	23,472	2	2.1	—	—	—
w^m	—	—	—	69,302	0	2.9
ec	17,676	0	1.6	57,323	0	2.4
cv	16,460	2	1.4	—	—	—
ct	12,914	0	1.1	57,323	1	2.4
v	29,384	2	2.6	61,119	1	2.6
m	—	—	—	39,923	2	1.7
g	12,914	0	1.1	57,323	4	2.4
f	34,811	8	3.0	130,421	15	5.5
car	—	—	—	69,302	1	2.9
Total	216,437	19	19.0	713,001	30	30.0
	$\chi_1^2=15.80$, d. f.=9, P=.08, $\chi_2^2=27.24$, d. f.=9, P=.002					
	$\chi_1^2+\chi_2^2=43.03$, d. f.=18, P<.001					
ru	12,755	0	0.6			
h	27,155	1	1.2			
th	5,681	0	0.2			
st	27,155	1	1.2			
p^p	21,474	4	1.0			
cu	5,681	0	0.2			
ss	21,474	0	1.0			
sr	5,681	0	0.2			
e^s	27,155	1	1.2			
ca	5,681	0	0.2			
Total	159,892	7	7.0			
	$\chi_3^2=11.50$, d. f.=9, P=.25					

* TIMOFEËFF-RESSOVSKY's data are based on two or more experiments having different exposures to X-rays. Doses in Mutationsforschung in der Vererbungslehre 3,900 and 5,000 r.

tains a χ^2 which is significant at the five percent level of significance. This is not due merely to the omission of small numbers from the calculations as is shown in table 12 where more than one-third of the χ^2 was contributed

by the control data. The results of this further analysis suggest the possibility that the two species, *funebris* and *melanogaster*, may react differently to the X-rays even though their normal rates of mutation are apparently quite similar. The data on the normal rates of mutation for each of the two species being considered are too scant to allow a more definite conclusion to be drawn.

The next type of data to be considered is from two sources—(1) TIMOFEËFF-RESSOVSKY (1937) and (2) JOHNSON and WINCHESTER (1934)—and is on the subject of reverse mutations. These data include both v and c types of observations.

TABLE 14

Summary of all mutations in different directions within the white series of Drosophila melanogaster, produced by X-ray treatment (dosage approximately 4,800 r).

ORIGINAL GENE	MUTATED TO							TOTAL
	w	w^{bf}	w^{e}	w^{a}	w^{b}	w^{z}	W	
W, wild	25	1	3	1	2	5		48,500
w^{co}, coral	1							6,000
w^{b}, blood	3		1					12,000
w^{e} cherry	1							5,000
w^{a}, apricot	2		1					11,000
w^{e}, eosin	13				1	2	2	39,000
w^{bf}, buff	1							5,500
w^{t}, tinged	1							7,000
w, white		1	1		1			54,000

The conclusions to be drawn from table 13 are essentially those of the corresponding analyses of our own data with the exception that the "*f*" locus now appears to possess the higher rate of mutation in the JOHNSON-WINCHESTER data. It was found that 24.74/43.03 of the combined χ^2 for the sex-linked genes was contributed by the "*f*" locus.

If one uses TIMOFEËFF-RESSOVSKY'S data to test the rate of reverse mutation on chromosome I against that rate on chromosome III, he obtains $\chi^2 = 1.93$, d. f. $= 1$, $P = .17$. This is essentially the same result as that obtained from our own data for direct mutations.

TIMOFEËFF-RESSOVSKY'S study (1933) of the mutation rates of the alleles in the white locus of Drosophila is adapted to the χ^2 test in interpreting the significance of the observed differences.

Two questions will be asked about the data of table 14: (1) Is the rate of change of the different alleles to the "white" gene fundamentally the same for all alleles? To test this question, we take the white mutations in the first column aside from the "white" gene itself and test the eight remaining alleles for homogeneity of mutation rate. The eight observation classes give 134,000 observations with 47 mutations, or an average rate of

0.00035. Seven degrees of freedom are available for deviations from expectation. One finds that the χ^2 is 7.55 and is associated with a probability of .35. There seems to be nothing in the above data to indicate that the different alleles are significantly different in their rates of mutation to "white." (2) Are the frequencies with which the various alleles change to genes other than "white" fundamentally different? To answer that question, we test the homogeneity of the rates of mutation for all nine genes, omitting the data of column one. There are 188,000 observations and 22 mutations, which gives an estimated mutation rate of 0.00012. Eight degrees of freedom are now available, χ^2 is 11.8, and P is .16. It thus appears that no significant difference in rate of mutation to genes other than "white" is indicated by these data.

SUMMARY

The data obtained from irradiation research on visible gene mutations follow binomial distributions which are defined by probabilities less than 0.01. HOEL (1938), SUKHATME (1938), COCHRAN (1936), NEYMAN and PEARSON (1931), and FRYER (1940) have given evidence to show that the tabular χ^2 test is valid and useful for such data even when the expected numbers are as low as 0.5.

New data were obtained on visible mutations at 19 loci on the first three chromosomes of *Drosophila melanogaster*. These data are analyzed by the χ^2 test on the basis of these hypotheses: (1) that the genes within the sets observed have the same basic mutation rate, (2) that the mutation rate at a specific locus is directly proportional to the dosage of radiation applied, (3) that in so far as the sets of genes used are representative of their respective chromosomes, the fundamental rates of mutation on these chromosomes are the same, and (4) that for a particular gene and a fixed dosage, the wave-length of X-ray used does not affect the mutation rate. Our data indicate that the first hypothesis is not adequate to explain the variation observed when the "*ct*" locus and possibly the "*dp*" and "*px*" loci, are among those observed; but the other hypotheses fit the data quite satisfactorily.

Other sets of data were analyzed by χ^2 as illustrations of methodology. These data were on the following subjects: (1) continuous versus interrupted irradiation (from PATTERSON 1931), (2) reverse mutations (from TIMOFEËFF-RESSOVSKY 1933), (3) the production of mutations by heat (from BUCHMANN and TIMOFEËFF-RESSOVSKY 1936), (4) comparisons of mutation rates among the species of Drosophila: *funebris*, *melanogaster*, and *simulans* (from TIMOFEËFF-RESSOVSKY 1937), and (5) comparison of mutation rates at different alleles in the "white" series of *Drosophila melanogaster* (from TIMOFEËFF-RESSOVSKY 1933).

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IMMUNOGENETIC STUDIES OF SPECIES: QUALITATIVE DIFFERENCES IN THE SERUM OF BACKCROSS PROGENY FOLLOWING A GENERIC CROSS IN BIRDS[1]

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MOST geneticists feel that the mechanism of heredity is reasonably well understood, and many are now using genetics as a tool to investigate other biological problems. Among other fields, that of "speciation" is at the moment one of particular interest. That most species, if not all, differ in the constitution of their proteins has been deemed probable since the classical studies of NUTTALL (1904) and those of many later workers. For references to papers which cite numerous works of this general nature, see a previous paper (CUMLEY and IRWIN 1942). Such studies have given rise to the concept that for two related species, the proteins of each have *species-specific* as well as *common* constituents. The differences between the proteins of two closely related species are as yet detectable only by immunological methods, although REICHERT and BROWN (1909) used the technic of crystallography to establish differences in the hemoglobin of various animal species.

The rapid early growth of the knowledge of hereditary processes and the acknowledgment of its undoubted effect on an understanding of evolutionary processes caused LOEB in 1917 to raise the question as to whether the species-specificity (of proteins) was a Mendelian character. If not, he proposed that the possibility remained that Mendelian mutations may not have been the only essential factor in evolution.

A previous report (CUMLEY and IRWIN 1942) has presented experimental evidence of the segregation of one or more species-specific components of Pearlneck (*Streptopelia chinensis*) serum following backcrosses to Ring dove (*St. risoria*) of the species hybrid and backcross hybrids from the mating of Pearlneck and Ring dove. From these results, it was inferred that one or more genes on one or several Pearlneck chromosomes have effects on components of the serum species-specific to Pearlneck. The genes affecting these serum components seemingly acted independently of those producing cellular antigens which also were specific to Pearlneck.

Experimental evidence substantiating the above findings on the segregation of serum components is given in this report, for another species cross.

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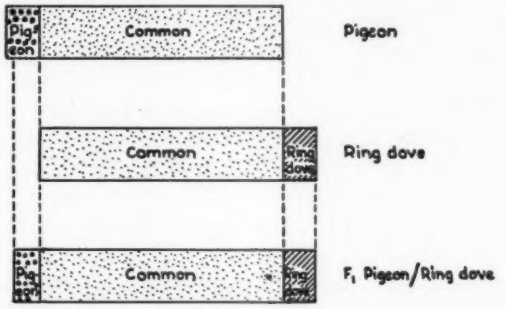
MATERIALS AND METHODS

Hybrids have been obtained by mating the domesticated form of the common pigeon (*Columba livia*) to Ring dove. We are very much indebted to PROFESSOR L. J. COLE and DR. N. L. CUTHBERT, under whose supervision many of these hybrids were produced, for the free use of serum and cells from the various hybrids and to DR. RAY D. OWEN, who was able to obtain hybrids from the mating of a Ring dove male to a pigeon female (by artificial insemination), as well as from the reciprocal mating. Twenty species hybrids were used in the course of these experiments. Two backcross offspring, $D234J_3$ and E387T, were obtained by mating species hybrids to Ring dove.

Diagram of the biochemical composition of the sera of Pigeon, Ring dove and their hybrid.



Note:-The proportions of specific and common substances are arbitrary.

Essentially the same technics were followed as are described in previous reports (CUMLEY and IRWIN 1940, 1942). Briefly, antisera were obtained from rabbits injected intravenously with the serum (plasma from heparinized blood) of pigeon or Ring dove. The precipitin tests were done by placing antiserum to a height of six to ten mm in each of a series of capillary tubes of about two mm diameter and then carefully layering above this the antigen (serum from the species or individual hybrids) in successive dilutions. A precipitate or "ring" at the interface indicated that an interaction had occurred between the antiserum and the antigen, while the lack of a definite precipitate was interpreted to mean the absence of a reaction.

The absorptions of antibodies were done by mixing undiluted antiserum to one species with given quantities of serum of the other. In the early part of this experiment the mixture was stored at 2 to 5°C for 24 hours, centrifuged, and the supernatant fluid was tested for reaction with the absorbing serum. The process was repeated until the absorption was complete. More

recently, the mixture of antiserum and approximately half the quantity of antigen required for absorption was kept at room temperature for about an hour and centrifuged. The supernatant fluid was then mixed with a small amount of antigen, stored overnight at 2-5°C, and the supernatant fluid was tested for a precipitate with the antigen. Small amounts of antigen were added to the supernatant until all the antibodies for the absorbing serum were exhausted. The necessity of avoiding overabsorption has been emphasized in the preceding report (CUMLEY and IRWIN 1942).

RESULTS

The interactions of the various antisera, against the respective sera of pigeon, Ring dove, and the species hybrid (F_1 Pgn./R.D.), with the different kinds of serum are given in table 1. From these results, it may be noted that the untreated antisera reacted with the same, or practically the same, dilution of sera from pigeon, Ring dove, and their hybrid, and no differentiation was possible between them by such tests.

But when the antiserum to pigeon serum was absorbed with Ring dove serum, it provided a "test-fluid" or "reagent" which reacted strongly with pigeon serum, definitely but less strongly with the serum of the F_1, and not at all with Ring dove serum.

Each of ten anti-pigeon sera when so absorbed produced a precipitate (the precipitate is composed of both antibody and antigen) with pigeon serum and not with that of Ring dove, although there were differences in the degree of reactions of individual antisera with pigeon serum. On the other hand, of a total of 19 anti-Pearlneck sera, following absorption by Ring dove sera (CUMLEY and IRWIN 1942), four reacted not at all and five only weakly with Pearlneck (the homologous) serum. Since Pearlneck and Ring dove are both species of the genus Streptopelia and pigeon belongs to a different genus (Columba), greater difficulty would be anticipated in effecting a differentiation of the proteins of the more closely related species, Pearlneck and Ring dove. That is, if there is a correlation of protein similarity with relationship, the proteins of pigeon and Ring dove should be less closely related than are those of Pearlneck and Ring dove, and antibodies for such differences should more readily be produced in rabbits.

The interaction of pigeon serum with the above reagent is presumably by virtue of one or more antigens of pigeon serum for which antibodies have been engendered in the rabbit and which are not removed from the antiserum by absorption with Ring dove serum. These antigens, then, represent a distinct antigenic difference of pigeon serum from that of Ring dove—that is, antigens of the serum species-specific to pigeon as compared with Ring dove.

Since the serum of the F_1 birds precipitated the specific reagent, one may

TABLE 1

Precipitin reactions of the sera of pigeon, Ring dove, their species hybrids and backcross hybrids.

ANTI-SERUM NUMBER	ANTISERUM	ABSORBED BY SERUM OF	ANTIGENS: HIGHEST DILUTION GIVING REACTION				
			PIGEON	RING DOVE	F_1-PGN./R.D.	$D_{234}J_3$	E387T
302S2	Pigeon	—	8,000	8,000	8,000		
302S2	Pigeon	Ring dove	1,024	0	32–128		
331S1	Pigeon	—	16,000	16,000	16,000		
331S1	Pigeon	Ring dove	80–256	0	32		
139S3	Ring dove	—	8,000	16,000	8,000		
139S3	Ring dove	Pigeon	0	1,024	128–1,024		
139S3	Ring dove	F_1-Pgn./R.D.	0	0	0		
240S3	Ring dove	—	16,000	16,000	16,000		
240S3	Ring dove	Pigeon	0	512+	64–512+		
240S3	Ring dove	F_1-Pgn./R.D.	0	0	0		
170S3	F_1-Pgn./R.D.	—			10,240		
170S3	F_1-Pgn./R.D.	Pigeon	0	256–512	128–256		
170S3	F_1-Pgn./R.D.	Ring dove	16	0	8–16		
170S3	F_1-Pgn./R.D.	Pigeon+Ring dove	0	0	0		
302S2	Pigeon	Ring dove	1,024	0	32–128	8	64
302S2	Pigeon	Ring dove+$D_{234}J_3$	512	0	16+	0	16+
302S2	Pigeon	Ring dove+E387T	32+	0	32+	16	0
331S1	Pigeon	Ring dove	80–256	0	32	16	16
331S1	Pigeon	Ring dove+$D_{234}J_3$	128	0	16	0	32
331S1	Pigeon	Ring dove+E387T	32	0	4	8	0
331S1	Pigeon	Ring dove+E387T +$D_{234}J_3$	32	0	0, 8*	0	0
296S1	Pigeon	Ring dove	16+	0	4	16+	16+
296S1	Pigeon	Ring dove+E387T	8	0	0	4	0
296S1	Pigeon	Ring dove+$D_{234}J_3$ +E387T	16+	0	0	0	0

The figures represent the highest dilution of the respective sera at which a precipitate was observed in combination with the particular antiserum. A zero (0) signifies that no reaction occurred in the lowest dilutions of serum.

* In two tests with the serum of the hybrid parent (E21Y) of E387T no observable precipitate was produced by this reagent, and only a doubtful reaction at a third trial at dilutions of 1:2 and 1:4. However, with the sera of certain other species hybrids, a definite ring was always noted.

conclude that at least a part of the antigens peculiar to pigeon are present also in the species hybrids. Reactions following the absorption of anti-pigeon serum by serum of the species hybrids would be the most precise

means of determining whether the serum antigens of the hybrids contained all or only a part of those particular to pigeon. These tests are being carried out and appear to show individual differences between the hybrids, but irrespective of whether the female parent was pigeon or Ring dove. The detailed results will appear later. In brief, the serum of each species hybrid seemingly contains nearly all the antigens of pigeon serum, and the variation between hybrids may be due to heterozygosity of the pigeon parents.

By the same kind of procedure outlined above, it has been demonstrated that Ring dove antiserum contains antibodies for species-specific antigens of both Ring dove and F_1 serum. The interaction of the specific reagent for Ring dove serum was usually at an appreciably lower dilution of F_1 serum than with that of Ring dove. However, since the absorption of antiserum to Ring dove by F_1 serum completely removed all trace of reaction for both Ring dove and pigeon sera, the only reasonable conclusion to be drawn is that the serum of the F_1 individuals by this test is indistinguishable from, and possibly identical with, the serum of Ring dove.

As shown in the table, an antiserum to F_1 serum contained antibodies to at least a part of the specific fractions of both pigeon and Ring dove. That is, this antiserum after absorption with pigeon serum, gave precipitates with both Ring dove and F_1 serum. The dilutions of pigeon and F_1 serum, at which reactions occurred with the reagent obtained by absorbing the antiserum with the serum of Ring dove, were somewhat lower (1:16) than were noted when these sera were tested with the reagent from anti-pigeon serum. Whether this peculiarity was by virtue of the antibody response of the individual rabbit or to a difference in either or both immunizing and binding properties of the serum of the F_1 is unknown. No reactive antibodies remained for the F_1 serum in this antiserum after absorption with both parental sera, hence by this test there was no evidence of a "hybrid substance" in the serum, although such a substance is found in the cells of all these species hybrids (IRWIN and COLE 1936b).

These experimental results are depicted diagrammatically in figure 1. Each species is represented as having an antigenic pattern in common with the other species and a part specific to itself. The proportions of the common and particular antigens are assigned arbitrarily, the only criterion for these proportions being the dilution at which the serum of one species gave precipitates with its antiserum before and after absorption by the serum of the other species.

The serum of the species hybrid is shown to possess all the common components of the parents, all those peculiar to Ring dove, and not quite all those specific to pigeon. Assuming for the moment that both common and specific components in these species are hereditary, then in the species

hybrid, possessing the haploid number of chromosomes of the pigeon and Ring dove, the haploid number of chromosomes—hence the genes acting singly—has the same effect upon the antigens specific to Ring dove, and practically the same effect in the pigeon, as the diploid number in either species. This parallels a similar finding for the components of the serum of the hybrid of Pearlneck and Ring dove (CUMLEY and IRWIN 1942) and for the cellular characters of various species hybrids (IRWIN and COLE 1936a, b; IRWIN et al. 1936) in which the hybrids have been found to possess all or nearly all the antigens of both parental species.

If the species-specific antigens of the serum of pigeon are determined by genes, a genetic separation would be anticipated in the offspring of matings of the F_1 to Ring dove. Two kinds of backcross offspring would be expected if there were in the serum of the species hybrid but one component peculiar to pigeon, namely, those with and those without the antigen. Four kinds would be expected if there were two serum antigens, as A and B, independently inherited—namely, A and B together, A and B carried singly, and neither, the last group possessing only the components of Ring dove. The number of different kinds of backcross offspring would be 2^n, in which n represents the number of different characters of the serum, assuming no separation of linked genes.

Given in the lower part of table 1 are the reactions of the sera of two such backcross birds (D234J3 and E387T) with several different reagents. (Only five backcross individuals have been produced in this laboratory over a period of more than ten years, and only two of these were available for these tests. The species hybrids, predominantly males, are highly sterile.) The serum of each backcross hybrid gave a precipitate with reagents for the specific pigeon antigens (that is, anti-pigeon sera Nos. 303S2, 331S1, and 296S1, each absorbed by Ring dove serum), showing that at least a part of the antigens specific to pigeon were present in the serum of each bird. The serum of D234J3 reacted at a somewhat lower dilution (1:8) than did that of E387T (1:64), with the reagent prepared from antiserum No. 302S2. With the reagents from antisera Nos. 296S1 and 331S1, however, the sera of both backcross hybrids gave strong precipitates at the same end-dilution (1:16).

Each of two antisera (Nos. 303S2 and 331S1) was then absorbed by the combination of the serum of Ring dove and D234J3, and this reagent from both antisera reacted definitely with the serum of pigeon at a high dilution, with that of the F_1 (unfortunately the F_1 parent of D234J3 was not alive), and also with that of the other backcross bird, E387T, but not with the respective absorbing sera.

Also, the serum of Ring dove and E387T were used together to absorb

each of these two pigeon antisera, and the resulting reagents gave a definite precipitate with the serum of pigeon, with that of the F_1 parent (E21Y) of E387T, and with that of D234J3.

From these results one may conclude that the serum of D234J3 has at least one component specific to pigeon which differentiates it from the serum of E387T. Likewise, the serum of E387T has at least one specific pigeon constituent which distinguishes it from the serum of D234J3. There is, of course, the possibility that the sera of these two birds may share one or more species-specific components, but this cannot be determined definitely unless and until backcross offspring can be obtained from either or both. There seems little chance of such an event. All these tests have been duplicated with serum from clotted blood from the respective individuals, with no discrepancies from the results obtained with p'asma.

To test whether the components of the two backcross birds equalled those of the hybrid parent (E21Y) of E387T and of the pigeon, an absorption of anti-pigeon serum No. 331S1 was made using the combination of sera from Ring dove, D234J3 and E387T. The reagent so produced gave a precipitate with pigeon serum, but only a faint if any precipitate in one test out of three with that of the F_1 parent (E21Y) of E387T. The same results were obtained following the absorption of anti-pigeon serum No. 296S1 with the combination of the three sera.

Thus, in minimum terms, if the serum of D234J3 contains a single antigen specific to pigeon, and but one other is present in the serum of E387T, the serum of E21Y possesses probably no more than these two components, while the serum of pigeon has at least one not found in the serum of E21Y nor in either D234J3 or E387T. The reactions following absorptions of pigeon antiserum by the serum of E21Y substantiate the above findings and conclusion, for such a reagent precipitated the serum of pigeon, but gave no trace of reaction with the serum of either D234J3 or E387T. Thus, as far as can be determined by this technic, the hybrid parent (E21Y) of E387T contains in its serum the components of both D234J3 and E387T. And a third component in pigeon serum must be adduced to account for the difference between pigeon serum and that of E21Y. The possibility of additional antigens specific to pigeon cannot be excluded, but cannot be tested unless more backcross offspring are obtained.

The segregation of qualitatively different components of the serum in the two backcross birds is readily explainable on a genetic basis, otherwise it is not at all understandable. That is, the antigens species-specific to pigeon are gene-determined, and segregation occurred in Mendelian fashion as a result of the backcross to Ring dove.

It is of considerable interest to know whether the specific pigeon antigens

of the serum of these backcross birds parallel in relationship the specific antigens of the blood cells. The cells of D234J3 have been reported (IRWIN and COLE 1936) to possess only a part of the total complex of cellular characters specific to pigeon. Accordingly, the necessary tests were made of the antigenic relationships of the cells of these two birds, with results as given in table 2.

From the interactions given in table 2, it may be noted that the cells of E387T also contain a part of the cellular pattern specific to pigeon. Further, following the absorption of anti-pigeon serum by the cells of both Ring dove and E387T, agglutinins remain in the reagent for the cells of the hybrid parent (E21Y) of E387T and for those of D234J3 as well. On the other hand, the absorption by the cells of Ring dove and D234J3 remove from the antiserum all the agglutinins at that dilution for the cells of E387T, but not for those of the species hybrid, agreeing in this latter reaction with the previous tests (IRWIN and COLE 1936b).

TABLE 2

Segregation of cellular components specific to pigeon.

IMMUNE SERUM	ABSORBED BY CELLS OF	AGGLUTINATION OF THE CELLS OF			
		RING DOVE	$F_1 \frac{\text{PIGEON}}{\text{RING DOVE}}$	D234J3	E387T
Pigeon	Ring dove	0	9	6	4
Pigeon	Ring dove and D234J3	0	7	0	0
Pigeon	Ring dove and E387T	0	7	4	0

Symbols: Zero (0) represents no agglutination at the first, or absorbing dilution. The digits represent the highest dilution of reagents at which agglutination of the particular cells was observed. The first dilution was one part serum in 90 parts of saline, then 1 = 90, 2 = 180, 3 = 360 . . . 9 = 23,040; the dilution always doubling.

Thus of the cellular antigens, the cells of E387T contain only a part of those which are present in the corpuscles of D234J3, and none other than this part. This relationship therefore holds for the causative genes, that is, D234J3 has the same genes, producing cellular characters specific to pigeon, as does E387T, plus one or more in addition.

But at least a part of the serum components specific to pigeon were qualitatively different in each of the two backcross hybrids, and the two antigenic systems are therefore not parallel. Independence in action of the genes for specific Pearlneck antigens of the serum and blood cells, respectively, has been shown in backcross individuals following the mating of Pearlneck and Ring dove (CUMLEY and IRWIN 1942), and the present evi-

dence substantiates the previous finding. The possibility still remains for other species of a parallelism between the occurrence of species-specific antigens of the blood cells and serum, and presumably therefore for the causative genes, either by virtue of linkage or multiple effects, if the latter are possible.

DISCUSSION

The findings from two different species crosses agree in showing conclusively that for these species the species-specificity (of proteins) falls within the pattern of Mendelian inheritance. Presumably one may anticipate that all species-specificities will be found to be gene-determined, and the question raised by LOEB (1917) is therefore answerable by experimental methods.

However, as pointed out by LANDSTEINER (1936) and later by the authors (CUMLEY and IRWIN 1942), there still remains the question as to whether or not protein differences between individuals may be found. If individual differences in proteins are not detectable, one can hardly escape the conclusion that protein differences of observable magnitude represent a line of demarcation between species and not between individuals.

Proteins are generally admitted to be highly important constituents of the "life-stuff" of living organisms. At the present writing, a study of protein differences between species and the search for individual differences will best be pursued by the technics of immunology, since direct chemical methods are not yet available which will accomplish these distinctions.

HALDANE (1938) has proposed that the final theory of evolution will be seen largely as a biochemical process, a view with which the authors are in complete accord. It would seem, then, that by the methods herein employed, one aspect of this theory is beginning to give way to an experimental approach.

SUMMARY

Antigenic components common to both species and others species-specific to each have been observed in the sera of pigeon and Ring dove, by immunological methods. The species hybrids contain all the common components and all or nearly all the specific components of both parental species. The respective sera of two backcross individuals, obtained from matings of species hybrid males to Ring dove, possessed one or more qualitatively different antigens specific to pigeon. This finding is in accordance with expectation on a genetic interpretation and provides evidence that genes influence the chemical constitution of proteins.

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SEXUAL ISOLATION STUDIES IN THE SPECIES COMPLEX DROSOPHILA VIRILIS[1]

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INTRODUCTION

SEXUAL isolation is a phenomenon frequently observed in the genus Drosophila. STURTEVANT (1921) reports that *D. melanogaster* males, while able to hybridize with *D. simulans* females, are more apt to mate with the females of their own species. LANCEFIELD (1929) found similar results in races A and B of *D. pseudoobscura*. DOBZHANSKY and KOLLER (1938) report cases of sexual isolation between *D. pseudoobscura* races A and B, *D. pseudoobscura* and *D. miranda*, and *D. azteca* and *D. athabasca*. MILLER (1939, 1941) finds that *D. athabasca* will form hybrids with both *D. affinis* and *D. algonquin*. His unpublished data suggest that sexual isolation exists between *D. athabasca* and both the other two species. Similar observations have also been made with forms of the *virilis* complex.

This complex at present includes three subspecies, or as some workers prefer to consider them, species. They are: *Drosophila virilis virilis* Sturtevant (STURTEVANT 1916), *Drosophila virilis americana* Spencer (SPENCER 1938), and *Drosophila virilis texana* Patterson (PATTERSON unpublished manuscript).

In order to study the sexual isolation in *virilis* and *americana*, SPENCER (1938) placed males with equal numbers of females of their own and the other subspecies and found that in both cases the males inseminated more of their own females than those of the other subspecies. He also found that the *americana* males more frequently inseminated *virilis* females than did the males in the reciprocal cross. PATTERSON, STONE, and GRIFFEN (1940b) made up series of single pair matings within and between the two subspecies. Their results showed that the cross-matings in which *virilis* was the female were more often successful than the reciprocal matings, thus corroborating SPENCER's results. Here, however, in most cases the success of the mating was not measured directly, but by the offspring obtained, so that many factors besides sexual isolation were involved.

The results of SPENCER and PATTERSON, STONE and GRIFFEN suggest the existence of sexual isolation in the *virilis* complex similar to that found by DOBZHANSKY and KOLLER in *D. miranda* and *D. pseudoobscura*. In order to get more critical data on sexual isolation between *virilis* and *americana*, and in order to ascertain whether there is any sexual isolation among

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the different strains of *americana*, further experiments were carried out. It is the purpose of this paper to report these experiments.

The author wishes to express his appreciation to DR. CURT STERN for his helpful advice and guidance throughout the problem. Appreciation is also due to DR. W. P. SPENCER for his friendly suggestions and unfailing cooperation in supplying many of the *americana* strains used. To DR. DONALD CHARLES the author is grateful for his criticism and help in regard to the mathematical treatment of the data.

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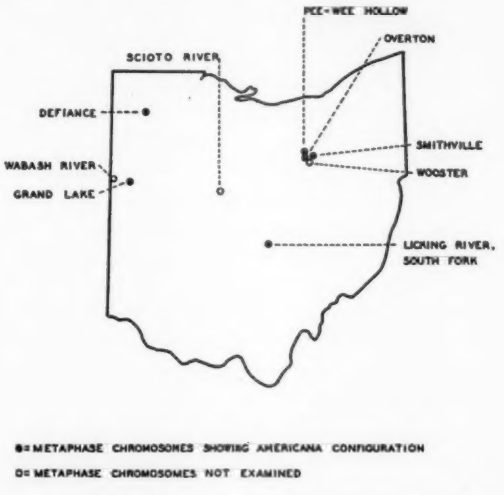

FIGURE 1. Localities in Ohio where *D. virilis americana* has been collected.

STRAINS OF VIRILIS AND AMERICANA USED

The wild strains of *virilis* and *americana* used are listed below, together with the sources from which they were obtained. Figure 1 indicates the localities in Ohio from which the *americana* strains were collected.

D. virilis virilis Sturtevant. This is the so-called Pasadena strain of *D. virilis*. It originated from a single pair which were bred from fruit exposed by DR. A. H. STURTEVANT at COLUMBIA UNIVERSITY in 1913. DR. STURTEVANT believes that it may have been crossed with some other *virilis* strain some time in the past. This strain will be referred to as Pasadena *virilis*, or simply as V.

D. virilis americana Spencer, from Smithville, Ohio. This is the type strain of *americana*. It is descended from a single fertilized female taken by

DR. W. P. SPENCER in the vicinity of Smithville, Ohio, June 25, 1936. This is one of the strains used in the early work on the subspecies (SPENCER 1938, 1940; HUGHES 1939; STALKER 1940; PATTERSON, STONE, and GRIFFEN 1940b). It will be referred to as Smithville *americana*, or As.

D. virilis americana Spencer from Overton, Ohio. This strain originated from a single fertilized female collected at Overton, Ohio, July 12, 1939. It will be referred to as Ao.

D. virilis americana Spencer, from Independence State Park, Defiance, Ohio. This strain is descended from a single fertilized female collected July 4, 1940. (The configuration of the metaphase chromosomes was determined through the kindness of DR. D. D. MILLER). It will be referred to as Ad.

D. virilis americana Spencer, from Licking River, south fork, near Buckeye Lake, Ohio. This strain originated from a single fertilized female collected June 28, 1940. It will be referred to as Al.

Americana is one of the rarest of the *Drosophila* species collected in Ohio. At many collecting stations it has not been taken at all, even after daily collections extending over a month or more. In a short collecting trip made during the summer of 1940 only 17 specimens of *americana* were taken among 15,171 specimens of other *Drosophila* species. Thus *americana* formed little more than one-tenth of one percent of the population which came to the traps. Little is known concerning the food of Drosophila in nature. However, judging from the rapidity with which *americana* comes to traps containing fermenting food in the laboratory, its rarity in such traps in nature probably indicates relatively small populations rather than a predilection for other food.

Americana has also been collected in Texas, and specimens which appear to be *americana*, but which have not been cytologically examined, are recorded from Tennessee.

SPENCER (1940) reports that *virilis* has been taken three times in the United States: in New York City; Terre Haute, Indiana; and Los Angeles, California. PATTERSON, STONE, and GRIFFEN (1940b) report this species from Texas and Louisiana. KIKKAWA and PENG (1938) report that it is common in Asia.

The collection records of PATTERSON, STONE, and GRIFFEN (1940b) give an average of one *virilis* in 20,000 specimens of the other *Drosophila* species, and their figures for the "red group," which contains both *texana* and *americana*, is one in 100,000. Since *americana* is apparently much rarer in Texas than is *texana*, this would indicate that *americana* is present in much smaller numbers in Texas than in Ohio.

SPENCER (1938, 1940) has listed many of the morphological and physiological characteristics by which *americana* and *virilis* differ. Table 1 gives the differences mentioned by SPENCER together with some additional ones discovered by the author which are marked by asterisks.

TABLE 1

Morphological and Physiological Differences between virilis and americana.

	VIRILIS	AMERICANA
	Morphological Differences	
1.	abdomen squattier	abdomen more fusiform
2.	body color lighter	body color darker
3.	eye smaller and lighter in color, eye pile coarse	eye larger and darker colored, very fine pile
4.	cheek wider	cheek narrower
5.	carina narrower	carina wider
6.	narrow cloud on post. cross-vein of wing	broad, lens-shaped cloud on post. cross-vein of wing
7.	fewer branches on arista	more branches on arista
* 8.	testis red	testis orange-red
* 9.	spermatheca longer and more rounded at tip	spermatheca shorter and squarer at tip
*10.	ventral sperm receptacle longer and thicker	ventral sperm receptacle shorter and thinner
11.	viscera tough, not easily ruptured	viscera rupture easily in dissection
12.	pupa case gray or black	pupa case reddish-brown
	Physiological Differences	
1.	etherizes slowly, recovers slowly	etherizes quickly, recovers quickly
2.	undisturbed adults occupy isolated positions in culture bottle	undisturbed adults form closely grouped aggregates
* 3.	young adults more active when disturbed	young adults more sluggish when disturbed
4.	puparia tend to form on sides of bottle	puparia tend to form in or on food
* 5.	females begin to lay eggs three to six days after eclosion	females begin to lay eggs four to nine days after eclosion
* 6.	period from fertilization of egg to eclosion shorter	period from fertilization of egg to eclosion longer

The chromosome configurations of *virilis* and *americana* have been investigated cytologically by HUGHES (1939), PATTERSON, STONE, and GRIFFEN (1940b) and genetically by STALKER (1940). These workers have shown that while both sexes of *virilis* have five pairs of rod-shaped chromosomes and a pair of dot-like microchromosomes, *americana* females have one pair of rods, two pairs of V-shaped chromosomes, and a pair of microchromosomes. *Americana* males have a pair of rods, a pair of V-shaped chromosomes, a pair of microchromosomes, and one V-shaped chromosome typically showing somatic pairing with two rod-shaped chromosomes. The pair of V-shaped chromosomes present in both sexes are autosomes, corresponding to chromosomes 2 and 3 of *virilis*. The V-shaped chromosome present once in the male and twice in the female corresponds to chromosomes X and 4 of *virilis*. The rod-shaped chromosomes pairing with this V-shaped chromosome in the *americana* male correspond to chromosomes Y and 4 of *virilis*. *Virilis* chromosome 5 corresponds to the rod-shaped chromosomes found paired in both sexes of *americana*.

METHODS

Two types of investigations were made. The first consisted of measurements of the sexual isolation as determined by the insemination frequencies. The second type consisted of a number of observations of matings between and within the two subspecies. These observations were made with the purpose of breaking down the phenomenon of sexual isolation into some of its component parts.

All flies used in these experiments were raised on cornmeal-molasses-Moldex food medium, made up with one-half the usual amount of molasses. It was found that while this decrease in molasses did not appreciably increase the length of the life cycle, it helped in keeping the cultures from becoming infected with bacteria, etc. This factor is more important in *virilis* and *americana* than in species such as *melanogaster*, since the former species require several days ageing before the adults lay fertilized eggs.

The flies to be tested were raised at 25 ± 0.75°C. When the adults in any one culture bottle began to emerge, the bottle was removed from the incubator and kept at room temperature until discarded. The room temperature averaged from one to four degrees lower than that of the incubator, and since adults age more slowly at a low temperature than they do at 25°C, the individuals emerging over a 24 hour period and ageing at room temperature were more nearly of the same physiological age than they would have been had they aged at 25°C.

Adults were collected every day between 10 and 12 a.m., just following the period of the day when most individuals seemed to emerge. Flies collected at this time were for convenience considered as 0 days old, although they were actually from 0 to 24 hours old.

Newly emerged flies were sorted for sex and aged at 25 ± 0.3°C. for a number of days. After ageing, the males and females were placed together without etherization in a culture bottle with fresh, heavily yeasted food. This culture bottle was kept at the same temperature for 24 hours, after which the females were dissected and the sperm receptacles examined for sperm. When it was inconvenient to dissect the females at the end of the 24 hour period, the bottles containing the males and females were placed in an electric refrigerator which ran at about 7°C, and kept there until the dissections could be carried out. Tests showed that neither *virilis* nor *americana* males inseminated their females at this temperature.

Dissections of females were carried out on a flat slide in physiological salt solution, and the sperm receptacles were examined under a compound microscope. It was found that contrary to DOBZHANSKY and KOLLER'S (1938) experience with *D. pseudoobscura* and *D. miranda*, inseminated *virilis and americana* females often contained sperm in one or both spermathecae, even when the ventral receptacles were empty. Thus a female was not considered uninseminated unless both spermathecae and ventral re-

ceptacle were seen to contain no sperm. As in *D. pseudoobscura* and *D. miranda*, the ventral receptacles of both *virilis* and *americana* virgin females contained long thread-like bodies which had a superficial resemblance to sperm, but which could be easily recognized with practice.

Virilis and *americana* arrive at sexual maturity sooner if aged under optimum food conditions than if partially starved during the ageing period. For this reason special care was taken to supply plenty of fresh food to maturing adults. In all cases where the ageing period extended for over three days, the culture bottles were changed every third day. Since the bottles were always heavily yeasted, such frequent changes did not entail any yeast starvation.

Sexual maturity of Drosophila males may be considered as that stage in their development in which they are able to inseminate females of their own species. However females of some Drosophila species pass through two distinct stages in their adult development. In the first stage they will mate, and can be inseminated, but are unable to lay any eggs due to the fact that their ovaries are undeveloped. In the second stage they can both mate and oviposit. In *virilis* and *americana* females these two stages are quite distinct. The term "sexual maturity" as used in this paper will indicate the first of these two stages of female development.

In order to determine the age at which the different strains used became sexually mature, equal numbers of males and females were aged separately for various periods, from one to six days, placed together for 24 hours, and the insemination frequencies of the females determined. In these experiments the males and females were always of the same age. Table 2 gives the results obtained.

TABLE 2

Insemination frequencies of females of different ages, exposed to males of their own strain and age for 24 hours. The numbers in parentheses give the number of females examined in each case.

STRAIN	AGE IN DAYS AT TIME OF MATING						
	1	2	3	4	5	6	7
V	0% (75)	0% (102)	1.4% (145)	89.5% (293)	97.8% (355)	100% (101)	
As	0% (56)	0% (83)	1.7% (116)	93.7% (300)	93.8% (397)		100% (55)
Ao		0% (47)	1.8% (57)	81.8% (77)	97.6% (281)	98.5% (65)	
Ad	0% (20)	0% (46)	11.9% (84)	85.0% (139)	96.8% (282)	94.9% (102)	
Al		0% (127)	6.6% (151)	72.1% (197)	95.0% (322)	95.8% (119)	

From the table it can be seen that there was no insemination in any strain up to the third day, with some insemination following the third day, and a good deal following the fourth. Since there was little increase in the percentage of females inseminated following the fifth day, five-day-old males and females were used in all subsequent sexual isolation experiments.

MATINGS WITHOUT CHOICE OF FEMALES

These experiments involved matings within the various strains of *virilis* and *americana*, between *virilis* and *americana*, and between different strains of *americana*.

TABLE 3

Insemination frequencies in matings within and between different strains of virilis and americana. Ten each of five-day-old males and females were placed in each bottle.

♀♀	♂♂	FEMALES INSEMINATED	FEMALES NOT INSEMINATED	PERCENT INSEMINATED	ISOLATION
V	V	347	8	97.7	
As	As	372	25	93.7	
Ao	Ao	274	7	97.5	
Ad	Ad	273	9	96.8	
Al	Al	306	16	95.0	
V	As	258	89	74.4	†
V	Ao	320	36	89.9	†
V	Ad	358	21	94.5	†
V	Al	282	52	84.4	†
As	V	8	316	2.5	†
Ao	V	44	315	12.3	†
Ad	V	40	334	10.7	†
Al	V	51	261	16.3	†
As	Ao	257	26	90.8	*
As	Ad	255	15	94.4	
Ao	As	290	20	93.5	*
Ao	Ad	297	20	93.7	*
Ad	As	277	18	93.9	
Ad	Ao	294	20	93.6	*

* Insemination frequency significantly lower than that of *one* of the intra-strain matings.
† Insemination frequency significantly lower than that of *both* of the intra-strain matings.

Males were placed with only one type of female and thus were unable to exercise any choice in mating. Bottles were made up of ten five-day-old males and ten five-day-old females of their own or another strain. At the end of 24 hours the females were dissected and examined for sperm. The insemination frequencies obtained are shown in table 3.

In experiments of this type, isolation between any two strains is shown only to the extent that the insemination frequency of a mating between them is lower than frequencies obtained in matings within each of them.

For example, table 3 shows that the insemination frequency for the mating between V females and As males is 74.4 percent. Before a comparison can be made between this figure and the frequencies of the inseminations within V and As, tests must be made to show that all three matings, V×V, As×As, and V×As, possess sufficient internal homogeneity to be treated as units. As an example of how these tests were made, let us consider the mating V×V. Here the distribution of inseminated females in all the bottles making up the mating was compared with the distribution that would be expected on a purely chance basis. The χ^2 test was applied to the observed and expected distributions, and the P value obtained in the case of V×V was .28, indicating that no significant difference existed between observed and expected distributions. Thus the mating V×V may be considered sufficiently homogeneous to be treated as a unit, and the χ^2 test may now be used to compare it with other matings. None of the P values obtained in the homogeneity tests was lower than .14, thus indicating that the χ^2 test may be used to measure differences between the matings shown in table 3. A P value of .05 or less was considered to indicate a real difference between any two matings.

The last column of table 3 indicates whether a given mating shows an insemination frequency lower than that obtained in one, (*); in both, (†), or in neither of the matings within the two strains involved. Only the matings marked (†) clearly indicate sexual isolation.

With these criteria of sexual isolation in mind, let us consider the data summarized in table 3. All matings between *virilis* and *americana* show sexual isolation. Also in all four *americana* strains tested, the matings with *virilis* in which *americana* was used as females showed much more complete isolation than the reciprocals. This is in agreement with the findings of SPENCER (1940).

The degree of sexual isolation shown in any of the interspecific matings depends on the *americana* strain used. Considering first those matings between *virilis* females and *americana* males, we see that the insemination frequencies range from 74.4 percent to 94.5 percent. Since none of the matings within any of the *americana* strains differ significantly from each other (with the exception of As×As and Ao×Ao, whose difference gives a P value of .035), we may conclude that the four *americana* strains have about the same inherent insemination ability. Thus significant differences shown in the V×A series of matings are probably not due to differences in the inherent insemination ability of the A strains, but actually represent differences in sexual isolation.

By applying the χ^2 test, it is found that each of the four V×A matings shows significant differences from all the others. In the reciprocal A×V series, there are fewer significant differences shown, and in two cases,

Al×V and Ao×V, and Ao×V and Ad×V, no significant difference in the insemination frequencies is found.

The lower part of table 3 shows the insemination frequencies obtained in matings among the different A strains. As the asterisks indicate, there were no clear cases of sexual isolation in intra-specific matings of this type.

A comparison of the V×A matings with the reciprocal series indicates that strains showing strong isolation in one cross may be relatively incompletely isolated in the reciprocal one. For example, on the basis of the cross V×Al, Al would be considered as showing relatively complete isolation, but in the reciprocal cross, Al×V, Al shows less isolation than any of the other three A strains.

Dobzhansky and Koller (1938) obtained similar results in matings between *D. pseudoobscura* and *D. miranda*. These workers found that although as a general rule matings between *D. miranda* females and *D. pseudoobscura* males gave the highest insemination frequencies, the outcome of one cross could not be predicted by a knowledge of the reciprocal one.

A comparison of the results obtained here with the geographical origin of the strains tested (see fig. 1) shows that while strains from different localities behave differently in crosses to *virilis*, a knowledge of the behavior of one strain does not allow one to predict the behavior of a strain from the same or an adjacent locality. For instance, As and Ao come from collecting stations eight miles apart, yet they differ more when crossed to V than do Ao and Ad, which come from stations separated by 134 miles.

MATING WITH CHOICE OF FEMALES

Where the sexual isolation between any two strains of Drosophila is relatively incomplete,—that is, when mating can take place between them almost as readily as within either of them,—then this very slight isolation may perhaps be most readily detected by experiments in which the males are given a choice of two types of females, one type being of their own strain, the other of an alien strain.

The above work involving matings in which the males were given no choice has shown that there exist many such cases of very slight isolation in matings within the *virilis* complex. In the experiments to be reported below, culture bottles were made up with two five-day-old males, ten five-day-old females of their own strain, and ten of an alien strain. The females were dissected and examined for sperm after a 24-hour exposure. The males were aged 20 in a bottle, the food being changed the third day. They were lightly etherized preliminary to being placed with the females. The females of the two strains, which were distinguished by small nicks on the costal vein of the right or left wing, were aged together under the same conditions as the males. Both sexes were kept at $25 \pm .3$°C throughout the age-

ing and mating periods. Since in these experiments the experimental (alien) and control (conspecific) females were aged together in the same bottle, the environmental conditions during the ageing and mating periods should not affect the degree of isolation observed.

In order to express more easily the degree of sexual isolation, an Isolation Index, suggested by DR. D. CHARLES, was calculated. This Isolation Index consists of the percentage of conspecific females inseminated minus the percentage of alien females inseminated, this number being divided by the percentage of conspecific females inseminated plus the percentage of alien females inseminated.

$$\text{Isolation Index} = \frac{\%\ \text{conspecific}\ ♀♀\ \text{insem.} - \%\ \text{alien}\ ♀♀\ \text{insem.}}{\%\ \text{conspecific}\ ♀♀\ \text{insem.} + \%\ \text{alien}\ ♀♀\ \text{insem}}.$$

If the males inseminate equal percentages of their own and the alien females, the index will be equal to zero, denoting no sexual isolation. If they inseminate only their own females, the index will be equal to +1, denoting complete sexual isolation. If, on the other hand, the males inseminated more of the alien females than the conspecific ones, the index would have a negative value.

Table 4 summarizes the results of matings between *virilis* and various strains of *americana*.

TABLE 4

Frequencies of intraspecific and interspecific inseminations in mixed cultures of virilis and americana.

♂♂	♀♀	% OWN ♀♀ INSEMINATED	% ALIEN ♀♀ INSEMINATED	χ^2 OF THE DIFFERENCE	P	ISOLATION INDEX*	N†
V	V & As	87.2	0	402.78	<.01	1.00	523
V	V & Ao	80.6	1.6	322.65	<.01	.96	518
V	V & Ad	63.6	19.2	144.44	<.01	.62	508
V	V & Al	90.0	1.2	411.59	<.01	.97	521
As	As & V	75.8	16.0	183.87	<.01	.65	510
Ao	Ao & V	75.1	16.9	178.03	<.01	.63	523
Ad	Ad & V	73.1	36.5	69.51	<.01	.33	515
Al	Al & V	64.1	39.5	32.12	<.01	.24	528

* See text.

† Total number of females examined. The alien and conspecific females were present in approximately equal numbers. Accidental deaths during the course of the experiment caused inequality in some bottles.

The χ^2 values in tables 4 and 5 were obtained by use of a formula derived from the four-fold table by DR. D. CHARLES.[1]

[1] $\chi^2 = NC^2N_F/N_U(4N_SN_D)/[N+C(N_S-N_D)]^2$. Where N = total number of females, both conspecific and alien. N_F = total number of conspecific and alien females inseminated. N_U = total number of conspecific and alien females not inseminated. N_S = total number of conspecific females. N_D = total number of alien females. C = isolation index.

From the data in table 4 it can be seen that there is in every cross distinct and significant sexual isolation between *virilis* and the *americana* strain involved.

DOBZHANSKY and KOLLER (1938) carried out similar experiments and found that in matings between *D. miranda* and *D. pseudoobscura*, in which the males were given a choice of both types of females, they began to fertilize the alien females after most of the conspecific females were fertilized. They explain their results as follows: "Two interpretations of the above results seem about equally plausible. First, the males may copulate with females of their own species so long as any of the latter remain unfertilized, and may turn toward females of the foreign species only after the supply of conspecific virgins is exhausted. The occasional females that are unfertilized by males of their own species even after two weeks' exposure may be those that have already used up the supply of sperm received during the first copulation, and have not yet remated. Second, our results may be described in terms of unequal probabilities of fertilization. Suppose that the probability of copulation taking place when males of the species A meet females of their own species equals a, and when they meet females of the species B equals b, (a > b). This being granted, the number of homogamic as well as heterogamic matings must increase as time goes on; some females of either species may remain unfertilized and others may be impregnated once, twice, three, and more times. Our data are not critical for a discrimination between the above alternatives."

The first of these alternatives—namely, the males inseminating alien females after the supply of conspecific virgins is used up—may apply to the matings reported here. If this is true not only for matings between, but within the two subspecies, then the measurements of sexual isolation obtained would vary with the inseminating ability of the males used. For example some males might inseminate not only all of their own, but likewise all of the alien females, in which case there would be no isolation shown. On the other hand, other males with less fertility, might inseminate only some of the conspecific and none of the alien females. In this case the isolation shown would be complete. Although no cases of the former type, where all 20 females were fertilized, were found, even an approach to this condition might have considerable effect on the results obtained. It is obvious that although in bottles where all or most of the females were inseminated the sexual isolation may be hidden, in no case will there be an apparent isolation shown where none actually exists. With these facts in mind, we may make comparisons between the different crosses reported in table 4, although all such comparisons must obviously be made with some reservations.

In all crosses between the two subspecies, those in which the male was *virilis* show a much more complete isolation than the reciprocal ones. This is in agreement with the results obtained in the crosses involving no choice of mate.

In these matings, as in those above, knowledge of the insemination frequency in any one cross does not allow one to predict the results of the reciprocal cross.

MATINGS BETWEEN DIFFERENT AMERICANA STRAINS

Table 5 summarizes the results of the experiments similar to those reported above, but involving only *americana* strains.

TABLE 5

Frequency of insemination in mixed cultures of americana, containing two types of females, one type from the same strain as the males, the other from an alien strain.

♂♂	♀♀	% OWN ♀♀ INSEMINATED	% ALIEN ♀♀ INSEMINATED	χ^2 OF THE DIFFERENCE	P	ISOLATION INDEX*	N†
As	As & Ao	70.2	67.5	.44	.51	.02	507
As	As & Ad	68.5	51.0	16.36	<.01	.15	509
As	As & Al	60.7	57.1	.71	.44	.03	551
Ao	Ao & As	64.7	49.8	11.63	<.01	.13	514
Ao	Ao & Ad	67.9	46.3	24.03	<.01	.19	547
Ao	Ao & Al	65.2	49.1	5.32	.02	.14	534
Ad	Ad & As	60.4	47.5	16.91	<.01	.17	477
Ad	Ad & Ao	55.3	51.0	.88	.36	.04	489
Ad	Ad & Al	62.6	57.3	1.58	.21	.04	538
Al	Al & As	72.0	59.6	9.27	<.01	.09	548
Al	Al & Ao	66.1	63.8	.29	.61	.02	522
Al	Al & Ad	60.3	52.4	4.10	.06	.07	552

* See text.

† Total number of females examined. The alien and conspecific females were present in approximately equal numbers. Accidental deaths during the course of the experiment caused inequality in some bottles.

In these matings the isolation indices are in no cases so large as those in the matings between the two subspecies. However, there appears to be significant isolation in six of the 12 matings, and with one exception every strain shows significant isolation from every other strain in at least one of the two reciprocal crosses. In the case of the exception, Al and Ao, in one of the reciprocal crosses (Al♂♂ × Al♀♀ & Ao♀♀), the P value is .06, which is on the borderline of significance. DOBZHANSKY and KOLLER (1938) report similar intraspecific sexual isolation in geographically separated strains of *D. miranda*.

It will be recalled that sexual isolation was not found in matings between *americana* strains in which the males were given no choice of mates. This indicates that where only slight isolations exist between two strains, the males show a preference for their own females if a choice is possible, but if they are given no choice, will mate with the alien females almost as readily as with their own.

MATING BEHAVIOR IN THE VIRILIS COMPLEX

In an attempt to gain some insight into the factors hindering insemination in crosses between *virilis* and *americana*, a series of observations was made on the copulations between and within the two subspecies. The Pasadena strain of *virilis* (V) and the Smithville strain of *americana* (As) were used throughout. Adult *americana* and *virilis* raised under comparable conditions are approximately the same size, and since individuals used came from well fed larvae, there was little if any size difference between the members of the two strains used.

Observations were made on single pairs, which had been aged for a number of days, on fresh food, heavily yeasted. The flies were etherized lightly, placed singly in creamers with fresh food for 24 hours; then each was transferred without etherization into a creamer with an individual of the opposite sex. The creamers were placed on a white sheet of paper, under a bright electric light, and kept at about 25°C throughout the period of observation. A hand lens was used in the course of the observations, which were made one at a time.

MATINGS OF VIRILIS FEMALES × VIRILIS MALES

Copulations were observed in 18 pairs of six-day-old flies. The typical mating behavior was as follows:

As soon as the male noticed the female he usually began to court vigorously. This courting consisted of licking her abdomen, especially the ovipositor, with his proboscis, and rubbing her abdomen with his fore-tarsi. At the same time he would frequently give quick sidewise flicks with one wing.

If the female ran away, the male was likely to lose her temporarily. Occasionally she would stand still, but would kick at the male with her hind legs. The male seemed to parry these kicks with his fore-tarsi. The female often gave flicks with her wings similar to those of the male, even while kicking. Finally, if the courtship was successful, the female would stand still, stop kicking, and spread her wings. The male then mounted almost immediately, and copulation occurred.

The average length of the intensive courting immediately preceding copulation was 9.3 seconds. The average length of copulation was two

minutes and 12 seconds, the shortest time being one minute and 15 seconds, the longest two minutes and 45 seconds. The range of duration of copulation is shown in table 6.

In 16 out of the 18 cases, mounting preceded copulation; in two cases copulation took place just before, or at the same time as mounting. STURTEVANT (1921) points out that in the genus Drosophila, copulation precedes mounting. Both subspecies of *virilis* studied seem to be clear exceptions to this rule. In the present writer's material no scissors movements of the wings was observed in either subspecies, although STURTEVANT reports it in *virilis*. Occasionally there were circling movements of the male around the female, but these were usually noticeable only in cases of prolonged courting.

TABLE 6

Frequencies of different copulation durations in matings within and between americana and virilis.

TYPE OF MATING	DURATION OF COPULATIONS IN SECONDS				
	40–60	61–90	91–120	121–150	151–190
V×V		1	3	11	3
As×As		3	3	4	1
V×As		2	4	9	5
As×V	2			1	

Copulation was usually terminated by the male withdrawing his penis but remaining mounted. The female then flicked her wings and kicked with her hind legs until he dismounted. In about half the cases the male began to court following copulation. This second courtship was less vigorous than the first and brought no response from the female other than kicking or running.

Dissections of 27 females, including the 18 mentioned above, showed that following a single copulation only 23 of the 27 were inseminated. There was no clear correlation between the length of copulation and the presence of sperm in the female. For instance the shortest copulation observed, 75 seconds in length, resulted in insemination of the female, while copulations of 115, 140, and 164 seconds did not.

Matings of americana females × americana males

Copulation was carefully observed in 11 six-day-old pairs, and 19 additional females were dissected after it was seen that they had copulated once. Of these 19, 14 contained sperm, five did not. Again there was no apparent correlation between length of copulation and presence of sperm. For example, two copulations of 95 and 155 seconds' duration, respectively,

resulted in insemination; on the other hand, two of 97 and 160 seconds' duration did not.

The behavior of the *americana* pairs was very similar to that of the *virilis* except in the following respects:

The courtship was distinctly longer than in *virilis*, the average time being 56 seconds. The average length of copulation was two minutes, the longest copulation being three minutes and 45 seconds, the shortest one minute and 30 seconds. The range of the duration of copulations is shown in table 6. The males seemed to have more difficulty in getting properly mounted than in *virilis*. If they mounted and could not copulate within two or three seconds, they were promptly dislodged by vigorous kicking and wing-flicking of the female. In most cases the females apparently attempted to dislodge the males 15 or 20 seconds before the end of copulation by means of kicking and wing-flicking. This rarely occurred in *virilis*.

Matings of virilis females × americana males

Copulation between *virilis* females and *americana* males was observed in 20 pairs which were six days old. Sperm was transferred in 13 of the 20 copulations. There seemed to be no clear correlation between presence of sperm and length of copulation. For example, copulations of 100 and 173 seconds' duration resulted in insemination, those of 90 and 173 seconds did not.

The courtship and copulation showed a number of characteristics not seen within either subspecies. The *americana* males paid much less attention to the *virilis* females than they normally did to their own females. Often they stood motionless, paying no attention to the females, even when the latter passed close by. In four cases the females courted the males first. They came up alongside the males and flicked their wings, and in two cases the females even rubbed the males' abdomens with their fore-tarsi. The reactions of the courted males varied considerably. In two cases they extended their fore or middle legs (a typical reaction of Drosophila when keeping other individuals away while they are feeding or resting). In no case did they immediately begin courting the females.

Courtship was often long and frequently interrupted. The average time for active courtship immediately preceding copulation was two minutes and nine seconds, much longer than in conspecific matings of either *virilis* or *americana*. The males followed the females much less vigorously than in conspecific matings, and if they lost them often stopped moving, and only began courting again when the females ran by. The *virilis* females apparently resisted the courting of the *americana* males much more than they did that of the males of their own subspecies. This resistance took the form of prolonged and vigorous kicking with the hind legs. In five cases the

males did not mount when the females spread their wings, but continued courting. The females closed their wings again in three to five seconds, but opened them repeatedly for short periods. In several cases the males attempted to mount after the females had partially closed their wings. In one case when the female spread her wings the male stopped licking, turned away, and began to feed.

When the males mounted they appeared to have difficulty in copulating, apparently because the females kept the tips of their abdomens close to the surface on which they were standing. If the males did not copulate within two or three seconds after mounting, the females kicked them off. There was an average of 2.9 mountings per copulation. One male mounted twenty-two times before copulating, and afterwards, being unable to separate from the female, was dragged, and part of the genitalia torn away. The average length of copulation was two minutes, the longest being three minutes and five seconds, the shortest one minute and ten seconds. The range is shown in table 6.

Matings of americana females × virilis males

Seventeen pairs of nine-day-old flies were observed for two or more hours each. The males in this cross courted rather sporadically and usually followed the females for only a centimeter or so. An approaching female was usually met by an avoiding reaction, such as an extended middle or hind leg, or a quick side-step. In some cases the males courted vigorously for twenty or thirty seconds and then stopped suddenly and ran off. The females usually ran and kicked vigorously, even after persistent courting.

In two of the three cases where copulation took place, the females spread their wings only slightly, and the males when in copula were too far back and had difficulty in staying mounted. The females kicked them off, but they copulated again almost immediately; the second time being further forward, they were not dislodged for forty-two and fifty-three seconds, respectively, although the females were kicking most of the time. The males did not court again, although subsequent dissection showed that there had been no insemination.

In the third case, although again the female did not spread her wings widely and kicked all during copulation, the pair was in copula for one minute and twenty-two seconds, and insemination took place.

The above factors which hinder the transfer of sperm between *virilis* and *americana* may be divided into two main groups: those mechanisms which are normally operative in intraspecific matings and those which are operative only in interspecific matings.

Among the first group may be mentioned such factors as copulation without insemination and resistance in the form of running and kicking by

which the females delay copulation. These factors are operative in both intra- and interspecific matings.

The second group includes such factors as the lack of coordination in copulation shown in the matings of *virilis* females to *americana* males. Here the males, although apparently less interested in the *virilis* females than those of their own subspecies, yet court fairly persistently. The females seem ready to mate, as is shown by their occasional courting of the males. Yet when the males actually begin to court, the females run and kick vigorously. On the other hand, when the females spread their wings, the males frequently fail to mount, an omission which was never observed in intraspecific matings. But the males may attempt to mount when the wings of the females are partly closed. When the males do mount, copulation is made difficult by the fact that they are frequently unable to reach the ovipositor plates of the female.

Likewise in the matings of *americana* females by *virilis* males, the sudden cessation of courting by the male and the kicking by the female not only during the courtship but also all during copulation have not been observed in either of the intraspecific matings.

Thus in crosses between *virilis* and *americana*, incomplete sexual isolation is produced partly by exaggeration of the normal mating behavior found in conspecific matings and partly by behavioristic peculiarities found only in interspecific crossses. These latter peculiarities seem to be in part associated with a lack of coordination between the male and female of the two subspecies.

DISCUSSION

Geographic isolation of two originally identical groups of animals may lead to such evolutionary divergence that if they come together again, any hybrids formed will be weak or sterile. Thus those members of the two groups which will interbreed to form such hybrids are at a selective disadvantage, and genes preventing such interbreeding would have a positive selective value.

Dobzhansky (1937a, b, 1940) suggests that "occurrence of hybridization between races and species constitutes a challenge to which they may respond by developing or strengthening isolating mechanisms that would make hybridization impossible." He believes that the genes producing these isolating mechanisms, having a positive selective value at the border zone between the two races and a neutral value within each race, would gradually diffuse from the border zone throughout. If we accept this hypothesis as of general validity, the different degrees of isolation shown by strains from different parts of Ohio might be explained as follows: since

virilis is either very rare in Ohio or absent altogether and is known to occur in Texas, the zone of hybridization between *americana* and *virilis* would presumably be somewhere south of Ohio. The isolation genes would then diffuse northwards, and by the time they reached Ohio they might become variously fixed in the small apparently discontinuous *americana* populations. These populations would be expected to differ by chance as to the number and kind of such isolating genes which they contained.

Another hypothesis might likewise postulate no genetic isolation at the start of divergent evolution and its acquisition in the course of time. However, according to this hypothesis the genetic isolating mechanisms would be acquired, not by the challenge of hybridization, but independently during evolutionary changes which result in increasing differences between sub-groups. "The evolutionary origin of interspecific sterility lies not at the beginning of divergent evolution but occurs in the course of it as a by-product" (STERN 1936). The data presented in this paper might equally well be interpreted according to this hypothsis. Even in the absence of *virilis*, insular *americana* populations might be expected to acquire isolating mechanisms by the chance fixation of certain genotypes. The different *americana* populations would then be expected to vary considerably in the strength of the isolating mechanisms which they acquired.

Since the different localities in Ohio are represented by only one *americana* strain each, the picture of the distribution of the isolation genes that is obtained is far from complete. Accordingly, these data are not critical for a discrimination between either of the above alternatives, or a combination of both.

There are many mechanisms which keep two groups of related organisms isolated. Among them DOBZHANSKY (1937a, b) mentions: geographical isolation, ecological isolation, seasonal or temporal isolation, sexual isolation, mechanical isolation, failure of sperm to reach or penetrate eggs, inviability of the zygote, and sterility of the hybrid. Thus sexual isolation is only one of the many ways the mixing of the *americana* and *virilis* genotypes may be inhibited. In addition to it, mechanical isolation has been observed in one case, V♀ × As♂ (see above). Finally SPENCER (1940) and PATTERSON, STONE, and GRIFFEN (1940b) report partial sterility of the hybrids and low egg hatchability in crosses between the two subspecies. Whether this low hatchability of the eggs is due to zygote mortality or to a large number of eggs remaining unfertilized is not known.

SUMMARY

The Pasadena strain of *Drosophila virilis virilis* was tested for sexual isolation with strains of *D. v. americana* from four localities in Ohio. In experi-

ments in which males of one subspecies are placed with equal numbers of females of the other, *virilis* shows sexual isolation in matings with all *americana* strains. The matings in which *virilis* enters as the male show significantly greater isolation than the reciprocal ones. The *americana* strains vary in regard to the sexual isolation they show when mated to *virilis*.

Similar "no choice" experiments show no sexual isolation among any of the four *americana* strains.

In experiments in which males are placed with equal numbers of conspecific and alien females, matings between the two subspecies show that the males exercise choice and inseminate more of the females of the conspecific than the alien strain.

Similar matings involving a choice of females demonstrate sexual isolation among all *americana* strains. This isolation is much less complete than that found between *americana* and *virilis*.

Strains of *americana* of the same geographical origin may behave differently in crosses to *virilis*. The degree of isolation existing among different *americana* strains in intraspecific matings does not appear to be correlated with the distance separating the localities from which they were collected.

Observations on pair-matings between and within the two subspecies show that the sexual isolation mechanisms in interspecific crosses depend on two types of behavioristic peculiarities. One type is normally found in intraspecific matings; the other type, found only in interspecific matings, is associated with lack of coordination between the male and female.

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STUDIES OF SPOTTING PATTERNS. V. FURTHER ANALYSIS OF MINOR SPOTTING GENES IN THE HOUSE MOUSE[1]

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INTRODUCTION

MOST of the larger spotting patterns of rodents have been found to depend on the interaction of a number of mutant genes. Such forms as the various grades of piebalds in guinea pigs, the Dutch and English patterns of rabbits, hooded rats and the many pied and variegated races of the house mouse appear to have been produced by fanciers who collected and combined by selective breeding the many mutant factors which had occurred. The evidence from experimental breeding as reported by CASTLE (1940), PUNNETT and PEASE (1925), WRIGHT and CHASE (1936), DUNN and CHARLES (1937), and DUNN (1937) leaves no doubt that in each species mutations with effects on white spotting have occurred at many loci. In very few cases has it been possible to isolate the effects of one or a few mutant genes; in general the analysis has to be carried out with complexes of genes from which the effects of individual ones have to be inferred. Moreover, in the guinea pig and mouse, where the breeding evidence is most adequate, a great deal of non-genetic variability accompanies the extensive white spotting patterns and obscures the smaller genetic differences. In spite of these difficulties several types of spotting genes and at least two types of gene interaction have been demonstrated.

Two types of spotting genes which have been identified may be designated as major and minor ones. This designation is preferable to the conventional antithesis of "main gene" and "modifiers," since there proves to be no sharp line between the latter categories. Major spotting genes are represented by the piebald (*ss*) mutations of guinea pigs, mice, and rats, which have relatively large effects on white spotting. Minor spotting genes are those with small individual effects, such as the "modifiers" of hooded spotting in the rat studied by CASTLE (1919), the genes with small effects found by WRIGHT and CHASE (1936) in the guinea pig, and similar ones found in the mouse by DUNN and CHARLES (1937). In the latter two cases the analysis showed that the minor genes produced spotting in the absence of the major ones, hence were not modifiers.

Of the interactions found the additive effect and absence of dominance of

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individual minor mutations in the guinea pig seem proved by the analysis of WRIGHT and CHASE (1936) and to be made probable for the minor pied mutations of the mouse (DUNN and CHARLES 1937). A different type of interaction was found to exist between another major mutation (*W*) of the mouse, leading to variegated spotting, and a complex of minor recessive mutations. The latter produce little or no effect except in the presence of the major mutation (*W*) and may thence be referred to as modifiers. The modifiers themselves probably interact additively with each other (DUNN 1937).

It is possible that these two modes of interaction are essentially the same, the "modifier" relation appearing when the effects of the minor factors are individually so small that the effect of a major factor is needed to bring them to the threshold of expression. The data needed for deciding this question would consist of measurements of the individual and combination effects of minor spotting factors.

MATERIALS

In a previous paper (DUNN and CHARLES 1937) a genetically new type of white spotting in the mouse was described and shown to be due to a collection of several spotting genes with small effects producing a belt of white around the body. The race containing this complex of genes (referred to collectively as "k" factors) did not contain the major spotting mutation *s s*. A partial analysis showed the number of spotting alleles with appreciable effects to be at least two, probably more; while the variance of F_2 suggested that a large number of factors with very small individual effects might be involved. The additional analysis reported below was intended to separate some of the constituent elements from the "k" complex.

DESCRIPTION OF THE "K" SPOTTED LINE

We first inbred the "k" line for an additional seven brother-sister generations beyond those previously reported. Beginning in the sixth generation, sub-lines were set up from the descendants of several F_5 brother-sister pairs. In each line brothers and sisters were mated without selection, although the spotting grades of the parents chosen indicate that these came chiefly from the center of the spotting range (2–30 percent of dorsal white). The estimates of dorsal white spotting of these lines (for methods of estimation see DUNN and CHARLES 1937, p. 20) are given in table 1.

The different families proved to be quite similar in the range of white spotting although there were some variations in the modal grade. Thus families 1a and 1c derived from family 1 were probably less spotted than the parent line. All lines produced small litters (a characteristic of the all-white line from which all were descended), and all were eventually lost, so

TABLE 1

Frequency distributions of amounts of dorsal white spotting in inbred lines of "k" spotted mice.

FAMILY	GENERATION	PERCENTAGE OF DORSAL WHITE								
		1–5	6–10	11–15	16–20	21–25	26–30	31–35	36–40	N
1	F_{6-12}	2	24	20	35	38	19	1	2	141
1a	F_{6-8}	3	21	10	14	18	1			67
1b	F_{6-10}	3	4	7	28	32	11	1		86
1c	F_{6-7}	5	11	10	3	1				30
1d	F_{6-12}	1	8	10	19	43	14	5	3	103
2*	F_{7-12}	1	6	13	16	14	8		1	59
3*	F_{7-11}	1	4	3	7	9	7	3		34
		16	78	73	122	155	60	10	6	520

* From different F_6 pairs of family 1.

that it was not possible to get a conclusive answer to the question whether genetic differences were present in the "k" line and were then sorted out in the different inbred families. It is likely, however, that such was the case.

In all of the inbred families the wide range of variability in amount of spotting persisted, even to the 13th generation. A high degree of nongenic variation is thus characteristic of this type of spotting, as of others.

RESULTS OF CROSSES OF "K" SPOTTED BY SELF

The largest inbred "k" family (family 1) was chosen as a source of animals for further study. The experiments were designed (1) to get an estimate of the number of genes with spotting effects by which the "k" line differed from an unspotted (self-colored) line and (2) to segregate these different mutant genes into separate lines where their individual effects might be observed. To these ends, "k" spotted individuals from the 11th and 12th generations of family 1 were crossed reciprocally with the dilute brown self stock described previously (DUNN and CHARLES 1937); F_1 animals were backcrossed to the self and to the "k" spotted parent families, an F_2 generation was bred, and matings were made between F_2 pairs from different parts of the F_2 range. From the latter, inbred lines arose which differed in amount of spotting and presumably contained different spotting genes.

Unfortunately only four lines survived to the seventh or later inbred generation, and this number was insufficient to permit a complete analysis of the many genes involved. The F_1, F_2, and backcross to the parent spotted line were repetitions of experiments previously reported. Although the previous experiments had shown that there were several spotting genes in the "k" complex, the number of them and their individual effects could not

be detected because of phenotypic variability and because the finer details of spotting were not followed. In the repetition we classified offspring as self-colored when they showed no trace of white spotting on the body or tails, as tail-spotted when they showed white spots on the tail only, and as belly-spotted when they showed a spot varying in size from a few white hairs to a large white spot. Foot spotting was not followed. For each of these spotted classes an estimate was made of the amount of white spotting on each individual. The tail has about 30 vertebrae and if entirely white was graded 30, if one-third white, 10, and so on. The amount of white on belly and back was estimated in percentage of the total ventral or dorsal surface. It was found that with few exceptions wherever the belly was spotted, the tail was also, and wherever the back was spotted, the belly was also, so that in the tabulations each animal may be entered only once—that is, under the class of maximum spotting. Thus those animals classified as belly-spotted are also tail-spotted, those classified as back-spotted are also spotted on belly and on tail.

The F_1, F_2, and backcross generations are shown in table 2. Two results are noteworthy. (1) All F_1 animals are spotted either on tail or belly or both, showing that some of the mutant genes of the "k" complex are dominant in the combination tested. (2) In the F_2 only 13 out of 191 animals (about 1/16) are entirely unspotted, suggesting that all of the spotted ones contain one or more dominant factors, at least one of which is necessary for the expression of any spotting. If two dominants are involved, then the unspotted animals should constitute 1/16 of the F_2. The fit to 15:1 ratio shows $\chi^2 = .108$ and $P = .75$. This would lead to an expectation of about one-fourth self-colored animals in the backcross of F_1 by self (dilute brown). The actual result was 42 selfs out of 122 offspring, which is a poor fit ($P = .02$). In the backcross the genes black and brown were also segregating, and while among the blacks, self and spotted forms appeared in about a 1:3 ratio (23:56, $P = .4$), among the browns there was a deficiency of spotted (19 self:24 spotted). Very small white tail spots on brown animals may have been overlooked. Other evidence makes it unlikely that brown acts as a suppressor of spotting. We may conclude that more than one, probably two, dominant factors with minor effects are present but the number is uncertain.

The number of spotting factors segregating in addition to these dominants is difficult to estimate. However, they must be numerous, for in an F_2 of 191 animals the degree of spotting characteristic of the "k" parental type was not recovered at all. The maximum extent of dorsal spotting found on any F_2 animal was less than the minimum found in the parental stock. The most probable interpretation is that a large number of factors, each with small effects, or no effects at all when alone, must be present

together with one or more dominants before the amount of spotting characteristic of the "k" parent is attained. This would indicate that the minor mutations are in general recessive, but the need for many to be present together would of course simulate recessiveness.

TABLE 2

Results of crossing "k" spotted by self-colored (dilute brown) mice.

GEN.	PARENTS	SELF	TAIL SPOT GRADES		BELLY SPOTTED PERCENTAGE WHITE			DORSAL SPOTTED PERCENTAGE WHITE						
			1–5	6–10	1–5	6–10	11–15	1–5	6–10	11–15	16–20	21–25	26–30	31–40	T
P_1	"k" spotted (F_{8-11})					—	—		13	12	20	30	12	3	90
P_1	self (dilute brown)	all*													
F_1	self×"k" spotted		15	4	28										47
F_2	$F_1 \times F_1$	13	68	6	78	4		22							191
BC	F_1×self	42	70	5	4			1							122
BC	F_1×"k" spotted				8	2	2	20	20	10	7	8	1		78

* Among several hundred such mice observed, three had a small white spot at tip of tail, probably nongenetic variants.

The backcross of F_1 by "k" spotted (last line table 2) yielded only spotted animals; all of these were spotted on the belly or back in addition to the tail. The modal grade of dorsal spotting was between one and ten percent, compared to 21–25 in the "k" parent race. The extreme extent of parental spotting was not recovered, although it is evident that some of the backcross animals contained combinations of minor factors which yielded the "k" phenotype.

The most interesting conclusions from the above are that most of the factors of the "k" complex have individually extremely small effects; only two of them show dominance.

SEGREGATION OF LINES WITH DIFFERENT GENES FROM THE "K" COMPLEX

If "k" spotting is due to the cumulative effects of several different genes, it should be possible by inbreeding to separate lines homozygous for different genes of this complex. Four such lines were established by mating together F_2 animals showing much, little, and no spotting, respectively, and continued by brother-sister matings. The results are shown in table 3. Family A began with matings of the F_2 animals which showed dorsal spotting. F_3 showed an increase in amount of spotting, and all offspring were spotted. Selection increased the extent of white spotting until in the seventh generation, when the line was lost, only animals with dorsal spotting ap-

peared. The average amount was less than in the original "k" line, but apparently many of the "k" genes had been recovered.

TABLE 3

Amount and location of white spotting in inbred lines derived from five different matings (A-E) of F_2 animals from the cross of "k" spotted by self.

FAM.	GEN.	SELF	TAIL ONLY	TAIL AND BELLY	DORSAL SPOTTING IN PERCENT					
					1–5	6–10	11–15	16–20	21–25	26–30
	F_2	13	74	82	22					
A	F_3		4	35	22	7	2			
A	F_{6-7}			1	15	13	6	12	2	2
B	F_3		4	20	38	6	1			
B	F_{8-11}				61	29	8	2	1	
C	F_{2-4}	3	10	5						
C	F_{5-13}	7	121							
E	F_{1-5}	105	4							

Family B likewise originated from dorsal spotted F_2 mice and produced only spotted animals, mainly with belly and small dorsal spots. The amount of spotting remained relatively uniform, the range and average being greater than in F_2, but less than in the "k" parent line.

Family C began with tail-spotted animals and with selection bred practically true to this condition, except for occasional self-colored animals which were probably non-genetical variants.

Family E was derived from F_3 self segregates. It bred true to this condition except for two animals with a few white hairs at tip of tail in F_5, probably non-genetical variants.

Thus, four different lines established from F_2 all showed different phenotypes, one approximating the original spotted line, one the self line, one showing tail spotting only, and one showing an intermediate amount of spotting.

All these lines were characterized by infertility and small litters, but it was possible to test two of them before they were lost.

INHERITANCE OF TAIL-SPOTTING

Animals from F_{10} of family C showing pronounced tail spotting only ($\frac{1}{3}$ to $\frac{2}{3}$ of tail white) were crossed with animals from a black self race which had never shown any white spotting. About three-fifths of the F_1 animals (table 4) showed a small amount of white at the tip of the tail,

TABLE 4

Tests of spotted mice from families extracted from cross of "k" spotted by self.

PARENTS	SELF	SPOTTED		
		TAIL	BELLY	DORSAL
1. tail spot (family C)×self (line 101)	23	32		
2. $F_1 \times F_1$ (from 1)	69	58		
3. dorsal spot (family B)×self (line 101)	59	15		
4. dorsal spot (family B)×self (family E)	15	37	20	2
5. all white×self (family E)			8	15
6. all white×self (line dbr)	15		33	3
7. belt spot (line 190)×self (line 101)	52	5*		
8. belt spot (line 190)×self (family E)	9	23†		

* Maximum spotting; tip of tail only

† Extensive tail spotting

never covering more than 1/10 of the tail; several of these showed also a small amount of mottling as small white spots below the tip. Since both parent races must have been largely homozygous, the variation in F_1 cannot be due to genetic segregation, but probably to minor chance individual differences. It is not surprising, therefore, that F_2 reproduces the character of F_1. Both self and tail-spotted animals appeared, the former in somewhat higher frequency than in F_1, the latter with very small amounts of spotting (not more than 1/6 of the tail). No simple interpretation fits the data. There are apparently mutant alleles leading to tail spotting in the tail spot family, wild type alleles of these being in the black self race. Animals heterozygous for the two types of alleles have about an even chance of showing a very small amount of white spotting. The wild type alleles are certainly not fully dominant and are probably different from the mutant allele producing tail-tip spotting found by GRÜNEBERG (1936).

TESTS OF EXTRACTED SPOTTED AND EXTRACTED "SELF" COLORED LINES

To test whether the self-colored line (family E) recovered in F_{3-8} from the cross of "k" by self was genetically like the self-colored parent line, it was crossed with several inbred lines known to contain recessive spotting factors. For comparison, animals from long inbred self-colored lines were crossed with the same spotted animals referred to above. The results are shown in table 4.

When tested by an extracted "k" line (family B), family E self animals gave mainly spotted F_1's (59 spotted:15 non-spotted), some of them with extensive spotting on belly and on back; family 101 self individuals crossed with the same animals of family B gave mainly non-spotted F_1's, with a

few showing only tail-tip spotting. The differences between these two F_1's were sharp and unmistakable.

In crosses with "all white" animals (containing the "k" complex added to *s s* pied), the family E selfs gave only spotted F_1's, most of them with dorsal spotting, while selfs from the dilute brown parent race gave unspotted animals as well, and the F_1 spotting was generally restricted to a small belly spot.

When the long inbred belted pied race (line 190) was used, most of the F_1's from family E showed extensive tail spotting; while those from the cross to line 101 self were nearly all unspotted.

Animals of the extracted self-colored family thus produce spotted offspring when tested by "k," pied, or combined "k" and pied genotypes; whereas unextracted self races do not do this or do it to a much less degree. This extracted self is therefore genotypically different from the parental self type. It seems most reasonable to assume that family E has lost some of its wild type alleles and that for these have been substituted mutant alleles from the "k" race. The latter appear to produce no spotting effect by themselves, but when combined with other spotting alleles, they interact with these to produce spotting.

Because of a disease epidemic, it was not possible to study the effects of these factors in combination in later generations. Their effects in combinations, however, were clearly of the "modifier" type, since unlike the previously recognized components of the "k" complex, they proved not to be spotting genes in their own right.

DISCUSSION

The phenotype known as "k" spotting, which consists typically of a white belt around the body, depends on the interaction of several mutant genes. After a cross to an inbred spotted line, the "k" complex may be broken up and parts of it recovered in extracted inbred lines. Some of the lines recovered after crossing are spotted only on the tail, others are characterized by dorsal spotting of various degrees, while others show no spotting but contain genes which show additive effects when combined with other spotting alleles. One or more of the mutants in the "k" complex show some dominance, since animals heterozygous for them show white spots on the tail. Most of the mutants are recessive and probably have little or no effect except when at least one or two other mutant genes (such as the above dominants) are present. The number of such mutant factors could not be determined accurately but is probably high (at least five with marked effects, probably ten or more in all).

Minor spotting factors of this sort have been reported frequently in the

house mouse and are probably common in both wild and laboratory stocks. In addition to the six occurrences cited by DUNN and CHARLES (1937), GRÜNEBERG (1936) has reported a recessive tail spotting, and BARROWS (1939) has studied a stock with tail spotting which he supposed to be due to interaction among several genes. In all cases the amount of spotting associated with any one gene is very small; interactions among the different genes have not been studied nor have they yet been combined with any of the major factors. In the guinea pig WRIGHT and CHASE (1936) have shown that the spotting phenotypes studied depend on many mutant genes. Only one of these (*s s*) has a major effect; the others have small individual effects which show in general little dominance or epistasis but which tend to be additive. The scheme which they have devised probably fits also the situation in pied mice in a general way.

Beyond these mutations with visible effects probably lie others which produce spotting only when several such are brought into combination with each other, or with other minor or major spotting genes. Two examples of such mutations have been found: one in the family reported in this paper which was extracted from a cross of "k" by self and which was itself unspotted but produced spotted offspring when crossed with spotted mice; the other instance was discovered (DUNN 1937) when self-colored mice (*w w S S*) from a selected stock of variegated (*W w S S*) were found to give spotted offspring when crossed with "k" spotted mice and were known to contain alleles of the so-called *m*(*W*) complex which by themselves do not lead to spotting. One may speak of the genes in these races as sub-visible or sub-threshold spotting alleles. In the case last cited, some of these alleles were taken from the self race, and by backcrossing and selection added to a collection of "k" genes. The race so produced had about 20 percent more white spotting than the "k" race without the *m*(*W*) alleles.

One may suspect that such sub-threshold alleles are common in laboratory stocks and in nature, although no careful search for them has been made. They are of great interest, for now that they have been found to interact cumulatively with both the additive "k" alleles and with the specific modifiers of the *m*(*W*) type, they may be viewed as bridging the gap between these two types of gene interaction. It may be assumed that the fundamental or usual type of gene interaction in spotting is additive or cumulative. Many mutations, however, produce so small a change in the reactions leading to spotting that the threshold of visible expression is not reached. If visible effects are produced only in the presence of a gene or genes which have caused a larger change in development, then the first type of mutation (sub-threshold) will appear to act as a specific modifier of the second.

SUMMARY

The spotted phenotype known as "k" has been inbred for 10–12 generations in several families and is shown to vary from about one to 40 percent of dorsal white, usually in the form of a belt.

It is shown to differ from the unspotted condition as found in a long inbred dilute brown line by a complex of mutant genes with small effects. One or more of these are dominant; several others are more or less recessive, all probably interact additively.

New inbred lines were derived from different parts of the F_2 from the above cross. Of these, two resembled the "k" parent but showed less spotting, one showed tail spotting only, and one showed no spotting. The latter proved to be different from the parental unspotted race and when crossed with recessive spotted races gave spotted animals in F_1.

It is suggested that this unspotted race contained mutant alleles which produced no spotting effects themselves but did produce spotting when combined with other spotting alleles. Such "sub-threshold alleles" therefore act as modifiers of other factors with more extensive effects by interacting additively with them.

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